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20-EPI-VITAMIN D_3 ANALOGUES: A NOVEL CLASS OF POTENT REGULATORS OF CELL GROWTH AND IMMUNE RESPONSES

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Abstract—The 20-epi-vitamin D_3 analogues are a novel class of vitamin D_3 derivatives, structurally related to 1α,25-dihydroxycholecalciferol (1α,25$(OH)_2D_3$). They are characterized by an altered stereochemistry at carbon 20 in the side-chain. *In vitro*, these new analogues were found to be considerably more potent as regulators of growth and differentiation in the human histiocytic lymphoma cell line U 937 than 1α,25$(OH)_2D_3$, despite a practically unchanged calcemic activity *in vivo*. The most potent analogue, KH 1060, inhibited cell proliferation by 50% at 10^{-12} M (14,000 times more active than 1α,25$(OH)_2D_3$). At the same time, KH 1060 induced cell differentiation at concentrations as low as 10^{-14} M. In addition, the 20-epi-vitamin D_3 analogues were found to be very potent inhibitors of T-lymphocyte proliferation induced by interleukin-1 or alloantigen. In this respect, they were several orders of magnitude more active than the potent immunosuppressive agent cyclosporin A (CyA). KH 1060, the most potent analogue, inhibited interleukin-1-induced mouse thymocyte proliferation by 50% at 3×10^{-16} M and allogeneic stimulation of mouse spleen lymphocytes at 5×10^{-15} M. These effects were considered to be mediated by inhibition of interleukin-2 release from activated T-lymphocytes. The new analogues are of potential interest in the prevention of graft rejection and in the treatment of psoriasis, cancer and auto-immune diseases.

In recent years, the physiologically active metabolite of vitamin D_3, 1α,25-dihydroxycholecalciferol [1α,25$(OH)_2D_3$], has achieved status as a hormone with effects on cell growth and cell differentiation, in addition to its well-known role in calcium homeostasis [1]. The effects of 1α,25$(OH)_2D_3$ are believed to be mediated via binding to a specific receptor that belongs to the family of nuclear receptors for glucocorticoids, estrogens, thyroxine and retinoic acid [2, 3].

The therapeutic potential of 1α,25$(OH)_2D_3$ and its synthetic analogue 1α-hydroxycholecalciferol has been investigated in cancer and psoriasis [4–6]. However, the strong calcemic activity of these compounds limits their clinical usefulness [7] and an intensive research activity has therefore been directed at finding new vitamin D analogues with a more favourable therapeutic profile. As a first step in this direction, the recent development of the new analogue calcipotriol (MC 903) [8–10] has led to an effective and safe topical treatment of psoriasis vulgaris [11, 12]. Other new analogues have been reported to increase survival of leukemic mice [13] and to regulate immune responses [14], with a decreased risk of inducing calcemic side-effects.

The present investigation reports the biological activities of a new series of vitamin D_3 analogues, characterized by an altered stereochemistry at carbon 20 (C20). These novel 20-epi-vitamin D_3 analogues were investigated *in vitro* for their effects on cancer cell growth and differentiation, using the human histiocytic lymphoma cell line U 937, which displays high affinity receptors for 1α,25$(OH)_2D_3$ [15]. These new analogues were also tested for their ability to affect calcium homeostasis *in vivo* after oral administration to rats and for their binding to the intestinal receptor for 1α,25$(OH)_2D_3$, which mediates calcium uptake from the intestine [1].

Finally, the 20-epi-vitamin D_3 analogues were assessed for their effects on T-lymphocyte activation *in vitro*. Previous studies have shown that monocytes, macrophages and activated lymphocytes possess receptors for 1α,25$(OH)_2D_3$ [16]. In analogy with the potent immunosuppressive agent cyclosporin A (CyA), 1α,25$(OH)_2D_3$ has been shown to inhibit proliferation of activated T-lymphocytes, presumably by interfering with IL-1/IL-2 and other cytokine-mediated functions [17, 18]. It was therefore considered of interest to investigate the effects of the new analogues on cytokine-mediated T-cell activation, in comparison with 1α,25$(OH)_2D_3$ and CyA.

MATERIALS AND METHODS

Chemicals. 1α,25$(OH)_2D_3$ and analogues modified in the side-chain were synthesized in the Department of Chemical Research, Leo Pharmaceutical Products. The methods of synthesis are described in the patent applications Nos. WO 89/10351, PCT/DK 90/00186, WO 90/09991 and WO 90/09992. Cyclosporin A (CyA) was a gift from Sandoz (Basle, Switzerland). The vitamin D_3 derivatives were dissolved in isopropanol or dimethyl sulphoxide, and CyA was dissolved in absolute ethanol for the *in vitro* studies. The solutions were stored at −20°. For *in vivo*

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studies, solutions were prepared in propylene glycol and kept at 4° for a maximum of 7 days.

Effects on cancer cells in vitro. Studies on cell proliferation and differentiation were performed using the human histiocytic lymphoma cell line U 937, as described previously [9]. U 937 cells were adjusted to 1×10^5 cells/mL in RPMI 1640 (Gibco, U.S.A.). The medium was supplemented with 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (FCS, Gibco). Cells (5 mL) were incubated for 96 hr at 37° in the presence of vitamin D_3 derivatives (10^{-14}–10^{-7} M). Control cultures received 0.2% solvent. At the end of incubation the cells were counted and assayed for survival by eosin Y exclusion. IC_{50} values were determined from the dose–response curves by graphical interpolation. Each compound was tested in three separate experiments, each experiment included controls with and without $1\alpha,25(OH)_2D_3$, and all cultures were performed in triplicate. A sample of cells from each culture was fixed with formalin and cell differentiation was assessed by staining for membrane-associated non-specific esterase by the method of Yam *et al.* [19]. The cells were scored under microscope and induction of cell differentiation was recorded as the lowest concentration of test compound giving a 3-fold increase in the number of esterase-positive cells, compared to control cultures (control cultures containing $6 \pm 2\%$ differentiated cells). Additional studies on cell proliferation were performed using the human breast carcinoma cell line T-47D (ATCC HTB 133) and the rat osteosarcoma cell line M1-OS/C42 (kindly provided by Dr T. Klenner, Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cells were adjusted to 5×10^4 cells/mL in medium RPMI 1640, as described above for the U 937 cells, and cultured for 7 days in the presence of $1\alpha,25(OH)_2D_3$ or KH 1060 (10^{-13}–10^{-7} M). Fresh medium and test compounds were added on day 4 of culture. Inhibition of cell proliferation was assessed by uptake of [^{3}H]thymidine ([^{3}H]TdR, 1 μCi/ml, 5 Ci/mmol, Amersham, U.K.) during the last 4 hr of culture. IC_{50} values were determined from the dose–response curves. Three separate experiments were performed, with each concentration tested in quadruplicate.

Effects on T-cell activation in vitro. Studies on T-cell activation were performed using the mouse thymocyte co-stimulatory assay [20] and the mixed lymphocyte reaction (MLR) between allogeneic mouse spleen cells [21]. Thymocytes were prepared from the thymus of 4–6-week-old Balb/c mice and adjusted to 5×10^6 cells/mL in RPMI 1640, supplemented with 5% FCS. Samples of 200 μL cells were cultured in the presence of interleukin-1 or interleukin-2 (IL-1, 2.5 units/mL; IL-2, 20 units/mL), Boehringer, Mannheim, Germany) and phytohemagglutinin-P (PHA, 10 μg/mL, Sigma Chemical Co., MO, U.S.A.) for 72 hr. For the MLR cultures, spleen cells were prepared from Balb/c and $CB6F_1$ mice and adjusted to 2.5×10^6 cells/mL. Cultures containing 100 μL of each cell suspension were incubated for 144 hr. Selected cultures were supplemented with high concentrations of IL-2 (250 units/mL). Solvent, vitamin D_3 derivatives or CyA were added at the start of incubation (max. solvent concentration 0.04%). Proliferation was assessed by [^{3}H]TdR uptake (1 μCi/mL) during the last 4 hr of incubation. Each compound was tested in three separate experiments and all cultures were performed in triplicate.

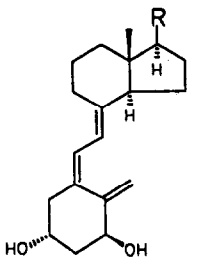
Effects on cytokine release in vitro. Mononuclear cells were obtained from the peripheral venous blood of healthy volunteers by density centrifugation with Lymphoprep® (Nycomed, Norway). For assay of IL-1 release, the cells were adjusted to 1.5×10^6 cells/mL in RPMI 1640 supplemented with 1% autologous human serum. The cells were stimulated with 2 μg/mL lipopolysaccharide (LPS from *E. coli*, Difco, U.S.A.) for 24 hr, in the presence of vitamin D_3 analogues. IL-1 in cell supernatants was determined by radioimmunoassay (RIA) (RPA 533, Amersham, U.K.). For assay of IL-2 release, mononuclear cells were adjusted to 2.5×10^6 cells/mL in RPMI 1640, supplemented with 2% FCS, and stimulated with 20 μg/mL PHA, in the presence of vitamin D_3 analogues, for 24 hr. IL-2 release was quantitated using cultures of $CD3^+$ T-lymphocytes, selected from human blood mononuclear cells by particle-bound anti-CD3 monoclonal antibody (Immunotech, France), as described by Leivestad *et al.* [22]. The specificity of the released IL-2 was verified by addition of antibodies to IL-2 (rabbit anti-human IL-2 polyclonal antibody, 50 μg/mL, Genzyme, U.S.A.). All cultures were performed in triplicate. Concentrations of vitamin D_3 derivatives resulting in 50% inhibition of cytokine release were calculated.

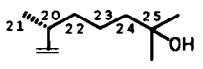
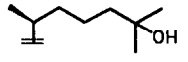
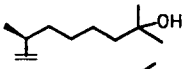
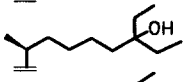
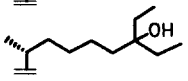
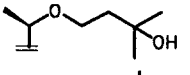
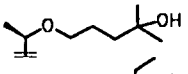
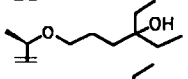
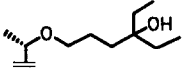
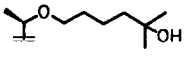
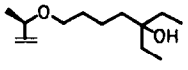
Receptor binding studies. Receptor protein from the intestinal epithelium of rachitic chickens was purchased from Amersham, U.K. (TRK.870). Samples of 500 μL were incubated with 10,000 dpm [^{3}H]$1\alpha,25(OH)_2D_3$ (180 Ci/mmol, Amersham, U.K.) and increasing concentrations of $1\alpha,25(OH)_2D_3$ or vitamin D_3 analogues were added. After incubation for 60 min at 22°, bound and free [^{3}H]$1\alpha,25(OH)_2D_3$ were separated on dextran-coated charcoal. The concentration resulting in 50% displacement of bound [^{3}H]$1\alpha,25(OH)_2D_3$ was calculated. Each compound was tested in three separate experiments.

Receptor protein was prepared from cultures of U 937, T-47D and M1-OS/C42 cells. Suspensions of 5×10^7 cells/mL were homogenized, sonicated and centrifuged at 30,000 *g* for 1 hr at 4°. The presence of the $1\alpha,25(OH)_2D_3$ receptor was checked by sucrose density gradient analysis as described by Colston *et al.* [23]. The supernatants were adjusted to 2 mg protein/mL and used for binding studies with $1\alpha,25(OH)_2D_3$ and KH 1060 as described above for the chicken intestinal receptor.

Effects on calcium metabolism in vivo. Calcemic effects of the vitamin D_3 derivatives were assessed in female, inbred Lewis rats (140–160 g), replete in vitamin D and receiving a standard laboratory diet containing 1% calcium and 0.75% phosphorus. Hypercalciuria was chosen as the most sensitive test parameter of vitamin D-like activity. Test compounds were given orally at 0.1, 1.0 and 10 μg/kg/day, for 7 days. Control rats received the vehicle (propylene glycol) and each experiment included a group treated with $1\alpha,25(OH)_2D_3$ (0.5 μg/kg/day). Each group

Table 1. Structure–activity relationship of 20-epi-vitamin D_3 analogues



Structure R	Compound code name	Inhibition of cell proliferation IC_{50} (M)	Induction of cell differentiation (M)	Receptor binding 50% displacement (M)	Calcemic activity relative to $1\alpha,25(OH)_2D_3$
	$1\alpha,25(OH)_2D_3$*	1.4×10^{-8}	2.0×10^{-9}	1.6×10^{-11}	1.0
	MC 1288	2.8×10^{-10}	7.5×10^{-11}	1.3×10^{-11}	2.3
	EB 1231	4.4×10^{-11}	1.0×10^{-11}	1.4×10^{-11}	5.0
	MC 1301	8.2×10^{-11}	1.0×10^{-11}	1.6×10^{-11}	1.2
	CB 966*	1.7×10^{-9}	4.0×10^{-10}	9.0×10^{-11}	0.2
	MC 1292	3.4×10^{-9}	5.0×10^{-10}	7.3×10^{-10}	0.1
	KH 1059	1.7×10^{-10}	1.7×10^{-12}	1.8×10^{-10}	2.7
	KH 1060	1.0×10^{-12}	1.0×10^{-14}	1.3×10^{-11}	1.3
	KH 1139*	1.4×10^{-10}	1.0×10^{-10}	5.0×10^{-11}	0.3
	KH 1049	4.2×10^{-11}	5.0×10^{-12}	4.5×10^{-11}	1.4
	KH 1067	5.0×10^{-11}	5.0×10^{-12}	2.6×10^{-11}	0.8

U 937 cells (1×10^5 cells/mL) were cultured for 96 hr in the presence of $1\alpha,25(OH)_2D_3$ or vitamin D_3 analogues (10^{-14}–10^{-7} M). All compounds were tested in three separate experiments. Cell proliferation was determined by cell counting and the inhibitory effects were expressed as IC_{50} values. Cell differentiation was assessed by the histochemical evaluation of membrane-associated esterase activity. Binding to the $1\alpha,25(OH)_2D_3$ receptor from rachitic chicken intestine was measured by displacement of [^{3}H]$1\alpha,25(OH)_2D_3$. Calcemic activity was measured in Lewis rats, as the increase in urinary calcium excretion from day 3 to 7 of a 7 day treatment period with $1\alpha,25(OH)_2D_3$ (0.5 μg/kg/day p.o.) or vitamin D analogues (0.1–10 μg/kg/day p.o.). The calcemic activity of the analogues was calculated in relation to that of $1\alpha,25(OH)_2D_3$. Calcium concentrations in urine were 18.4 ± 1.5 μmol/day in control rats and 89.1 ± 7.8 μmol/day in rats treated with 0.5 μg/kg/day $1\alpha,25(OH)_2D_3$ (N = 10, mean ± SEM).

* Reference compounds with normal stereochemistry at C20.

consisted of three rats. The rats were placed in metabolism cages and urine was collected daily. Calcium in urine was determined by complex formation with *o*-cresolphthalein. The urinary excretion of calcium was calculated from day 3 to 7 of the experiment (steady-state conditions). The dosage resulting in an excretion of calcium comparable to that measured in rats given 0.5 μg/kg/day of $1\alpha,25(OH)_2D_3$ was calculated from the dose–response curve and expressed in relation to $1\alpha,25(OH)_2D_3$. The choice of the $1\alpha,25(OH)_2D_3$ dosage of 0.5 μg/kg/day was based on a full-scale dose–response experiment (0.01–10 μg/kg/day $1\alpha,25(OH)_2D_3$).

RESULTS

Structure–activity relationship of 20-epi-vitamin D_3 analogues

Table 1 shows a series of new vitamin D_3 derivatives, characterized by an altered stereochemistry at C20. The human histiocytic lymphoma cell line U 937 was used to evaluate the effects of these derivatives on cell growth and differentiation. Upon incubation with $1\alpha,25(OH)_2D_3$, the rate of cell proliferation was decreased ($IC_{50} = 1.4 \times 10^{-8}$ M), and cell differentiation was induced (at 2.0×10^{-9} M).

20-Epi-$1\alpha,25(OH)_2D_3$ (MC 1288) was 50 times more active as an inhibitor of cell proliferation and 25 times more active as an inducer of cell differentiation than $1\alpha,25(OH)_2D_3$ itself. Compared to MC 1288, elongation of the side-chain led to a further increase in activity (EB 1231), whereas the introduction of an oxygen atom at position 22 decreased the activity (MC 1292). However, by increasing the side-chain length of MC 1292 and replacing the C26 methyl groups with ethyl groups, an analogue (KH 1060) which was 14,000 times more active than $1\alpha,25(OH)_2D_3$ in inhibiting U 937 cell proliferation was obtained. The shape of the dose–response curves for all the tested analogues was similar to that obtained with $1\alpha,25(OH)_2D_3$. The maximum inhibition of cell proliferation, compared to control cultures, was 80%.

In the same cultures, KH 1060 induced cell differentiation in concentrations as low as 10^{-14} M. Compounds with "normal" stereochemistry at C20 were always less active than the corresponding 20-epimers (compare MC 1288/$1\alpha,25(OH)_2D_3$, MC 1301/CB 966 and KH 1060/KH 1139). Elongation of the side-chains of KH 1059 and KH 1060 led to analogues with decreased activity (KH 1049 and KH 1067). None of the compounds affected cell viability, as established by eosin Y exclusion.

The binding affinity of most of the 20-epi analogues to the chicken intestinal receptor for $1\alpha,25(OH)_2D_3$ was close to that of $1\alpha,25(OH)_2D_3$ (Table 1). In order to investigate whether the 20-epi analogues might exhibit an altered binding affinity to $1\alpha,25(OH)_2D_3$ receptors in cell types other than epithelial intestinal cells receptor preparations were obtained from suspensions of human U 937 cells, human T-47D breast carcinoma cells and rat osteosarcoma M1-OS/C42 cells. The presence of $1\alpha,25(OH)_2,D_3$ receptors in these cells was verified by sucrose-gradient centrifugation with [^{3}H]-$1\alpha,25(OH)_2D_3$. Table 2 shows the results obtained with $1\alpha,25(OH)_2D_3$ and KH 1060. Both compounds exhibited similar binding affinity in all cell types, close to that observed with the intestinal receptor. KH 1060 was, however, considerably more potent than $1\alpha,25(OH)_2D_3$ in inhibiting cell proliferation in the tumour cell lines than $1\alpha,25(OH)_2D_3$ (Table 2).

The ability of 20-epi analogues to affect calcium metabolism *in vivo* was studied next. Table 1 shows that most of the analogues were comparable to $1\alpha,25(OH)_2D_3$ in their ability to increase urinary calcium excretion in rats during a 7 day dosing period. Measurement of serum calcium levels on day 7 showed the same relationship, but this parameter was less sensitive in detecting small changes in the vitamin D activity than the urinary calcium assay (results not shown).

Effects of 20-epi-vitamin D_3 analogues on T-cell activation

The release of IL-1 from LPS-stimulated human peripheral blood mononuclear cells was first investigated. IL-1 release was determined by RIA, based on the detection of IL-1β, the major form of IL-1 released from human monocytes [24]. No inhibitory effects of $1\alpha,25(OH)_2D_3$, CyA or any of the 20-epi-vitamin D_3 analogues were observed in this system, in concentrations up to 10^{-7} M (results not shown).

In contrast, $1\alpha,25(OH)_2D_3$, CyA and the 20-epi-vitamin D_3 analogues inhibited the proliferative response of mouse thymocytes stimulated with IL-1 and sub-optimal amounts of PHA. The 20-epi analogues were considerably more potent than the corresponding derivatives with normal stereochemistry at C20. KH 1060 was the most potent analogue, approximately 600,000 times more active than $1\alpha,25(OH)_2D_3$ (Table 3).

Interference with the IL-1 induced proliferative response may be due to a reduction in IL-2 production/release. To test this hypothesis, KH 1060, as the most potent compound, was assayed for its ability to inhibit the release of IL-2 from PHA-stimulated human peripheral blood mononuclear cells. Fifty per cent inhibition was observed with KH 1060 at 3×10^{-14} M, with $1\alpha,25(OH)_2D_3$ at 1×10^{-9} M and with CyA at 1×10^{-9} M (Fig. 1). Furthermore, thymocytes activated by IL-2 and PHA were not inhibited by $1\alpha,25(OH)_2D_3$ or by any of the 20-epi-vitamin D_3 analogues in concentrations up to 10^{-8} M (results not shown).

These results suggest that the 20-epi-vitamin D_3 analogues, in analogy with CyA, might be useful in suppressing T-cell activation signals and thereby prevent rejection of transplanted tissues. *In vitro,* the mixed lymphocyte reaction (MLR) mimics the proliferative response to foreign histocompatibility antigens that occurs during the process of allograft rejection *in vivo* [21]. Table 4 shows that the 20-epi-vitamin D_3 analogues in concentrations from 10^{-12} to 10^{-15} M inhibited allogeneic stimulation in cultures of spleen cells from Balb/c and $CB6F_1$ mice. KH 1060 was again the most potent analogue ($IC_{50} = 5 \times 10^{-15}$ M). $1\alpha,25(OH)_2D_3$ and CyA inhibited the MLR at 5×10^{-11} M and 4×10^{-9} M, respectively. Addition of IL-2 (250 units/mL) completely counteracted the inhibitory effects of KH 1060, $1\alpha,25(OH)_2D_3$ and CyA in the MLR.

DISCUSSION

The recent discovery of the ability of $1\alpha,25(OH)_2D_3$ to regulate growth and differentiation in many cell types, including cancer cells, epidermal keratinocytes and activated cells of the immune system [1], has set the stage for the development of a class of novel agents with potential usefulness in hyperproliferative and immune-mediated diseases. Ideally, such agents should possess potent effects as regulators of cell growth and differentiation at concentrations well

Table 2. Effects of KH 1060 on tumour cell proliferation and receptor binding: comparison with $1\alpha,25(OH)_2D_3$

Compound	Tumour cell line (species)	Inhibition of cell proliferation IC_{50} (M)	Receptor binding 50% displacement (M)
$1\alpha,25(OH)_2D_3$	U 937	1.4×10^{-8}	2.8×10^{-11}
KH 1060	Histiocytic lymphoma (human)	1.0×10^{-12}	2.5×10^{-11}
$1\alpha,25(OH)_2D_3$	T-47D	1.1×10^{-9}	5.5×10^{-11}
KH 1060	Breast carcinoma (human)	1.4×10^{-12}	5.0×10^{-11}
$1\alpha,25(OH)_2D_3$	M1-OS/C42	7.1×10^{-9}	1.6×10^{-11}
KH 1060	Osteosarcoma (rat)	8.9×10^{-13}	1.3×10^{-11}

$1\alpha,25(OH)_2D_3$ or KH 1060 (10^{-13}–10^{-7} M) was added to cultures of U 937 cells (1×10^5 cells/mL, culture time 96 hr) and to cultures of T-47D and M1-OS/C42 cells (5×10^4 cells/mL, culture time 168 hr). Proliferation was assessed by cell counting (U 937 cells) or [^{3}H]TdR uptake (T-47D and M1-OS/C42 cells). Receptor preparations were obtained from homogenates of the cells and receptor binding was measured by displacement of [^{3}H]$1\alpha,25(OH)_2D_3$. Results are the mean of three experiments.

Table 3. Inhibitory effects of selected 20-epi-vitamin D_3 analogues and CyA on murine thymocyte activation

Compound code name	Cell proliferation [^{3}H]TdR uptake IC_{50} (M)	Potency relative to $1\alpha,25(OH)_2D_3$
$1\alpha,25(OH)_2D_3$*	1.9×10^{-10}	1
MC 1288	2.6×10^{-14}	7308
EB 1231	5.0×10^{-13}	380
MC 1301	6.7×10^{-15}	28,358
CB 966*	1.0×10^{-11}	19
KH 1060	3.0×10^{-16}	633,333
KH 1139*	2.1×10^{-11}	9
CyA	7.5×10^{-9}	0.03

Thymocytes from Balb/c mice (5×10^6 cells/mL) were stimulated with IL-1 (2.5 units/mL) and PHA (10 μg/mL). $1\alpha,25(OH)_2D_3$, vitamin D_3 analogues or CyA were added and the cultures were incubated for 72 hr. Proliferation was assessed by [^{3}H]TdR uptake during the last 4 hr of incubation. Inhibitory effects were expressed as IC_{50} values (mean of three experiments). The potency of the vitamin D_3 analogues was calculated in relation to that of $1\alpha,25(OH)_2D_3$.

* Reference compounds with normal stereochemistry at C20.

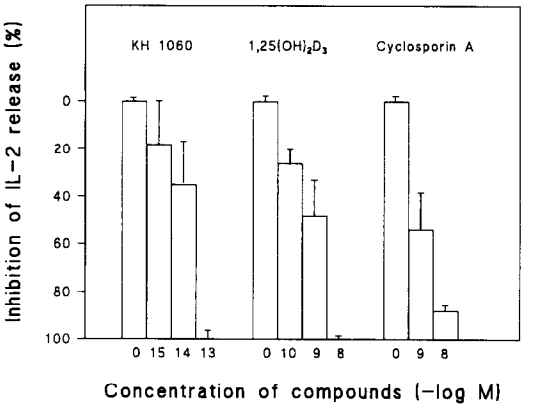


Fig. 1. Inhibitory effects of $1\alpha,25(OH)_2D_3$, KH 1060 and CyA on IL-2 release from PHA-stimulated human peripheral blood mononuclear cells (PBMC). PBMC were obtained by density sedimentation on Lymphoprep. The cells were adjusted to 2.5×10^6 cells/mL and incubated with PHA (20 μg/mL), and test compounds for 24 hr. Cell supernatants were assayed for IL-2 content, using purified $CD3^+$ T-cells obtained from human PBMC by binding to particle-bound anti-CD3 monoclonal antibody. All cultures were performed in triplicate.

below those that may induce side-effects related to the classical vitamin D activity on calcium absorption and bone mineralization.

In the present investigation, we have used the human histiocytic lymphoma cell line U 937 [15] to determine the effects of new vitamin D_3 analogues on cell growth and differentiation *in vitro*. This cell line has, along with the human promyelocytic leukemia HL-60 cell line, been used extensively in the study of structure–activity relationships of vitamin D metabolites and analogues [25]. In the presence of $1\alpha,25(OH)_2D_3$, the rate of proliferation of the U 937 cells is decreased and the cells differentiate along the monocyte–macrophage pathway, as assessed by the appearance of membrane-associated esterase activity. We have tested more than 200 new vitamin D_3 analogues in this system, over a period of 4 years. Analogues which were more potent than $1\alpha,25(OH)_2D_3$ were further assayed for their ability to bind to the $1\alpha,25(OH)_2D_3$ receptor, isolated from the intestinal epithelium of rachitic chickens, and for their ability to interfere with calcium homeostasis (induction of hypercalciuria in rats).

Using this approach, a series of new vitamin D_3 analogues with potent effects on cell growth

Table 4. Inhibitory effects of selected 20-epi-vitamin D_3 analogues and CyA on allogeneic T-cell activation

Compound code name	Cell proliferation [^{3}H]TdR uptake IC_{50} (M)	Potency relative to $1\alpha,25(OH)_2D_3$
$1\alpha,25(OH)_2D_3$*	5.2×10^{-11}	1
MC 1288	1.9×10^{-13}	274
EB 1231	3.2×10^{-13}	163
MC 1301	2.2×10^{-14}	2364
CB 966*	9.5×10^{-12}	5
KH 1060	4.9×10^{-15}	10,612
KH 1139*	2.7×10^{-12}	19
CyA	4.4×10^{-9}	0.01

Spleen lymphocytes from Balb/c and $CB6F_1$ mice were adjusted to 2.5×10^6 cells/mL. Mixed cell cultures containing 100 μL of each cell suspension were incubated for 144 hr. Addition of test compounds and assessment of proliferation were performed as described in the legend to Table 3.

* Reference compounds with normal stereochemistry at C20.

regulation *in vitro* were identified. These new analogues, characterized by an altered stereochemistry at carbon 20 in the side-chain (20-epi-vitamin D_3 analogues), were considerably more potent *in vitro* than $1\alpha,25(OH)_2D_3$. The reasons for this increased potency are not known. One possibility is that the new analogues may bind with higher affinity to the $1\alpha,25(OH)_2D_3$ receptor in cells responding to their growth regulatory activity than to the receptor in the cells that mediate calcium uptake from the gut. However, data obtained with the most potent analogue, KH 1060, showed that this analogue bound with similar affinity to receptor preparations from intestinal cells and from a number of cultured tumour cells (including the U 937 cells). It is therefore more likely that the increased potency is associated with post-receptor binding effects, such as increased metabolic stability of the receptor–drug complex or higher affinity for the DNA-binding site [26].

The 20-epi-vitamin D_3 analogues, when tested in rats to assess their effects *in vivo* on calcium metabolism, were found to exert calcemic effects comparable to those of $1\alpha,25(OH)_2D_3$. These findings correlate reasonably well with the binding of the various compounds to the intestinal receptor for $1\alpha,25(OH)_2D_3$, but are nevertheless surprising in view of their strong, direct effects on cell differentiation and cell proliferation. In order to establish the potential clinical usefulness of these new analogues, a number of additional studies on their effects on calcium metabolism are indicated. These include studies on calcium transport *in vitro*, using the everted gut-sac technique [27] and studies on bone calcium mobilization *in vivo* [28]. In addition, pharmacokinetic studies are needed to establish the rate of metabolism and the structure and activity of any metabolites.

In addition to their ability to regulate cell growth and differentiation of U 937 cells, the 20-epi-vitamin D_3 analogues were also tested for their effects on T-lymphocyte activation *in vitro*. This was done in comparison with the widely used immunosuppressive agent CyA, using test systems previously shown to be relevant in the study of the mechanism of action of this agent. These tests include measurement of the release of IL-1 and IL-2 from human peripheral blood cells and studies of T-cell activation by IL-1 or alloantigen in murine thymocytes and spleen lymphocytes [29].

In the present experiments, $1\alpha,25(OH)_2D_3$ and the 20-epi-vitamin D_3 analogues were not able to inhibit one of the first events in the processes leading to T-cell activation, namely the release of IL-1β from accessory cells. However, these results should be interpreted with caution as previous studies with $1\alpha,25(OH)_2D_3$ in the same type of test system have shown increased, decreased or unaffected IL-1 secretion [16, 30, 31], suggesting that small changes in the culture conditions may result in varying responses.

In contrast, CyA, $1\alpha,25(OH)_2D_3$ and the 20-epi-vitamin D_3 analogues inhibited IL-1 or alloantigen-mediated T-cell activation, the latter being by far the most potent inhibitors. No inhibition was observed in cells stimulated with IL-2. This type of response has previously been described for CyA and $1\alpha,25(OH)_2D_3$ [17, 29, 31, 32]. In addition, both CyA and the vitamin D_3 derivatives inhibited IL-2 release from stimulated T-cells. These findings suggest that despite very different types of receptors (cyclophilin for CyA [33] and the steroid-type receptor [2] for $1\alpha,25(OH)_2D_3$), the 20-epi-vitamin D_3 analogues exhibit a number of similarities with CyA. These effects may be mediated by inhibition of IL-2 production in T-lymphocytes, through regulation of gene transcription, as described previously for CyA and $1\alpha,25(OH)_2D_3$ [34, 35]. Alternatively, both types of compound may act via a calcium-mediated pathway. The early events in T-cell activation are calcium-dependent and CyA has been shown to inhibit calmodulin [36], whereas $1\alpha,25(OH)_2D_3$ is able to regulate intracellular free calcium levels [37].

In conclusion, we believe that the 20-epi-vitamin D_3 analogues, and in particular KH 1060, are amongst the most potent regulators of cell growth, differentiation and cytokine-mediated T-lymphocyte activation studied so far. Further studies are needed to investigate the effects of these analogues *in vitro* and *in vivo*. Preliminary experiments performed with i.p. administration of KH 1060 have shown that the compound significantly prolongs mouse skin allograft survival at doses as low as 0.02 μg/kg/day (J. L. Touraine and P. Veyron, Transplantation and Clinical Immunology Centre, Hôpital Edouard Herriot, Lyon, France) and prevents autoimmune glomerulonephritis in BN rats injected with $HgCl_2$ at 0.03 μg/kg/day (S. T. Lillevang, Dept. Immunology, Odense University Hospital, Denmark) (personal communications, manuscripts in preparation). These novel analogues may be of therapeutic interest in the prevention of graft rejection and in the treatment of psoriasis, cancer and autoimmune diseases.

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