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Cell Differentiating and Anti-proliferative Activity of Side-chain Modified Analogues of 1,25-Dihydroxyvitamin D_3*

Ewa Marcinkowska,[1] Andrzej Kutner[2] and Czesław Radzikowski[1]†

[1]Department of Tumor Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolf Weigl St. 12, 53-114 Wrocław, Poland and [2]Pharmaceutical Research Institute, Ludwik Rydygier St. 8, 01-793 Warsaw, Poland

Besides its calcium mobilizing activity *in vivo*, 1,25-dihydroxyvitamin D_3 has the ability to induce differentiation of human promyelocytic leukemia cells *in vitro*. We studied the cell differentiating activity of four novel analogues of 1,25-dihydroxyvitamin D_3, using the HL60 cell line as a model. We also analyzed the influence of these compounds on the proliferation of HL60 cells, normal human keratinocytes, normal fibroblasts from human skin and human keratinocytes transfected with human papillomavirus type 16. Two of the four analogues, i.e. those with extended side-chain, were found to display similar cell differentiating and anti-proliferative activities as 1,25-dihydroxyvitamin D_3. The other two analogues, with a shortened side-chain which included an additional hydroxyl, showed a substantially lower activity than that of 1,25-dihydroxyvitamin D_3. We observed distinct differences in sensitivity to the anti-proliferative activity of either 1,25-dihydroxyvitamin D_3 or its analogues between cells of different origin. **© 1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Since 1920, when vitamin D was discovered by Mellanby [1], the importance of this molecule for living organisms has become more and more evident. Initially emphasis was placed on the role of vitamin D in skeleton development alone, but further studies also revealed its role in endocrine homeostasis. Exposure of the skin to UV light was found to induce production of vitamin D, which is subsequently hydroxylated in liver and kidneys [2]. More recent data on the mode of vitamin D action have indicated a universal role of this molecule in the organism [3]. The hormonal form of vitamin D_3 (1,25-dihydroxyvitamin D_3) induces differentiation of promeyelocytic leukemia, breast cancer, colon adenocarcinoma, prostate cancer cells and normal or psoriatic keratinocytes *in vitro* [4]. The role of this physiologically occurring agent in an immune response is still far from being fully defined [5]. Potential application in oncology has attracted the attention of many researchers and has stimulated investigations into the inhibition of growth and induction of apoptosis in some cancer cell lines [6, 7]. The mechanism governing the beneficial effect of vitamin D_3 analogues after local application in the treatment of psoriasis [8, 9] is unknown and therefore enhances interest in the mode of action of vitamin D_3.

Various assays may be used in order to analyze the cell differentiating effect of vitamin D_3 analogues. Primary screening is usually performed *in vitro*, using the HL60 cell line as a model [10, 11]. The differentiation of the HL60 cells triggered by 1,25-dihydroxyvitamin D_3 resembles normal maturation of monocytes in bone marrow [12].

The purpose of our study was to compare the activities of selected vitamin D analogues, which have either relatively high or substantially lowered affinity for the vitamin D receptor (VDR). The structures of the studied analogues of 1,25-dihydroxyvitamin D_3 are presented in Fig. 1. Analogue 1 (previously developed [11] as a leading compound with separated calcium and cell differentiation activity) was subjected to a more detailed study of cell differentiation activity. To optimize the structure–activity relation we

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†Correspondence to C. Radzikowski. Tel. 7322 74; Fax: 6791 11; e-mail: radzikow@immuno.pan.wroc.pl

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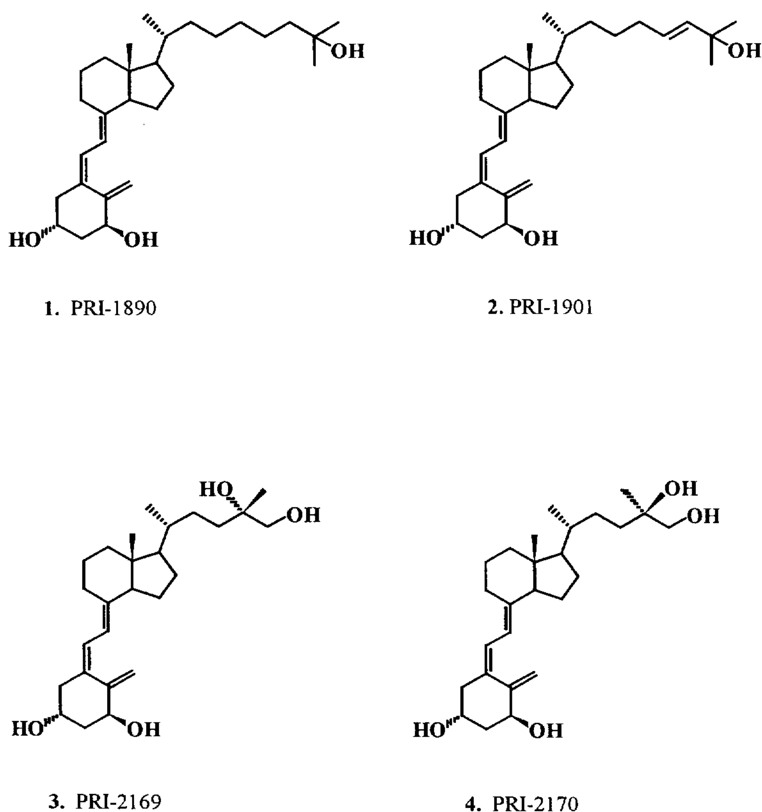

Fig. 1. The structures of 1,25-dihydroxyvitamin D_3 analogues studied.

evaluated the cell differentiation activity of analogue **2** with additional unsaturation in the side-chain at C-24a carbon atom. Compared to the affinity of 1,25-dihydroxyvitamin D_3 for VDR, that of analogue **1** is 140 times lower [11] and that of analogue **2** is 10 times as high [13]. Analogues **3** and **4** are new, differing from each other in the chirality center at the C-25 carbon involved in receptor binding. As compared to the structure of 1,25-dihydroxyvitamin D_3, they are additionally hydroxylated in the truncated side-chain at the terminal C-26. The affinities of analogues **3** and **4** for VDR are by three orders of magnitude lower than that of 1,25-dihydroxyvitamin D_3 (unpublished personal results).

It was interesting to study how the analogues influence the expression of the cell-surface markers on HL60 cells and the proliferation of human keratinocytes and fibroblasts from skin. Analogues with lowered affinity for VDR were tested for the influence of additional chirality and extra hydroxyl in the receptor binding site vicinity on the differentiation and proliferation of cells of different origin.

We tested the acquired phagocytic activity and the acquired nitro blue tetrazolium (NBT) reducing ability of differentiated HL60 cells. We also screened the increase in the expression of antigens typical of macrophages. For purpose of our study we selected CD11b, CD14 and the lactoferrin receptor. CD14 was described as a receptor for bacterial endotoxins, lipopolysaccharide (LPS) and LPS-binding protein (LBP) [14]. CD11b mediates phagocytosis in response to LPS [15]. The lactoferrin receptor is present on normal human blood monocytes, and lactoferrin stimulates the phagocytic cell function [16].

Another major objective of our study was to find out if the induced differentiation of HL60 cells would be accompanied by suppressed proliferation. And we, furthermore, intended to investigate if the proliferation of human skin cells would be altered by the compounds tested.

MATERIALS AND METHODS

Cells and culture conditions

Human promyelocytic leukemia HL60 cell line was obtained from European Type Culture Collection by courtesy of Professor Spik and Dr Mazurier (Laboratory of Biological Chemistry USTL, Lille). The cells were maintained in a suspension culture in an RPMI1640 medium supplemented with 10% fetal calf serum (FCS, North American Origin) (GIBCO, European Division), 100 units/ml penicillin and 100 μg/ml streptomycin (both from Polfa, Poland), and kept under standard cell culture conditions (humidified atmosphere of 95% air and 5% CO_2 at 37°C). For each series of experiments one vial of frozen cells was thawed.

Normal human keratinocytes and fibroblasts were isolated either from neonatal foreskins, or from plastic surgery skin samples, after overnight trypsinization at 4°C. Keratinocytes (passages 1–5) were cultured in Keratinocyte Serum Free Medium (GIBCO, European Division). Normal human fibroblasts (passages 1–10) were maintained in Opti-Mem (GIBCO, European Division) supplemented with 5% FCS. Human keratinocytes immortalized by transfection with human papillomavirus type 16 (d_2c cells, passages 78–90), were obtained from Professor Gangemi (Greenville Hospital System/Clemson University Cooperative Research and Education Program, Clemson, SC) and were cultured in the same medium as normal human keratinocytes. All the cells were maintained under standard *in vitro* culture conditions, as described above.

Compounds

1,25-Dihydroxyvitamin D_3 was obtained from Infarm, Warsaw. Analogues **1**, **2**, **3** and **4** (Fig. 1), labelled with codes PRI-1890, PRI-1901, PRI-2169 and PRI-2170 respectively, were synthesized in the laboratory of Dr Kutner at the Pharmaceutical Research Institute in Warsaw. The synthesis of analogue **1** was described earlier [17, 18]. An alternative method of synthesizing analogue **1** and analogue **2** was published elsewhere [13]. The synthesis of analogues **3** and **4** will be described in a separate paper. Samples of the compounds were stored in amber ampoules, under argon, at −20°C. The amount of each compound in the ampoule was determined by UV spectrometry (Carl Zeiss spectrophotometer, Jena) at 264 nm. Prior to usage, the compounds were dissolved in absolute ethanol to a concentration of 10^{-4} M, and subsequently diluted in culture medium to reach the required concentrations.

Antibodies

CD11b monoclonal antibodies (moAbs) (clone 44), CD14 moAbs (clone UHCM-1) and rabbit fractionated anti-lactoferrin antibodies were purchased from Sigma (St. Louis, MO) Fluorescein Isothiocyanate (FITC)-conjugated goat anti-mouse polyvalent antibody was also from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody was from Behring (Marburg). Antibodies were used at concentrations recommended by the suppliers. Mouse IgG1 and IgG2a were purchased from Bionetics (Bionetics Kensington, MD and Bionetics Charleston, SC). Normal rabbit Ig fraction was from DAKO (Glostrup).

Other chemicals

Iron-saturated lactoferrin from human milk and Propidium Iodide were from Sigma (St. Louis, MO).

HL60 differentiation assays

The cultured cells were seeded at the density of 1.75×10^5 cells/ml of culture medium on 24-well plates (Costar Cambridge, MA) to the final volume of 2 ml. The cells were exposed to either 1,25-dihydroxyvitamin D_3 or its analogues at concentrations ranging from 10^{-6} to 10^{-10} M for 96 h. Ethanol itself (a solvent for the compounds tested), in dilution corresponding to its highest concentration used for the compounds, produced no toxicity to all cell kinds studied and no differentiation of HL60 cells (data not shown). After 96 h of incubation, the cells were collected by centrifugation, washed in phosphate-buffered saline (PBS) and counted in a hemacytometer.

(A) To test the phagocytic capacity dried yeast *Saccharomyces cerevisiae* were boiled in PBS for 1 h, stained with trypan blue dye, washed twice, resuspended in PBS to the final concentration of $\sim 6 \times 10^9$ particles/ml and stored in a refrigerator. Prior to usage, 5 μl of the yeast suspension were mixed with 200 μl of RPMI1640 medium, supplemented with 20% FCS and 20% human type AB serum. The mixture was added to the pellet containing 6×10^5 HL60 cells and incubated at 37°C, for 3 h. The percentage of cells that had ingested the yeast was determined by counting in a hemacytometer. At least 200 cells/sample were counted.

(B) Superoxide production was assayed in an NBT reduction test. 200 μl of 0.1% NBT solution (NBT, POCH, Poland) enriched with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) were added to the pellet of 6×10^5 HL60 cells and then incubated at 37°C for 30 min. The percentage of cells containing black formazan crystals was determined by counting in a hemacytometer. At least 200 cells/sample were counted.

(C) To determine CD11b and CD14 expression by flow cytometry 2.5×10^5 HL60 cells in 50 μl of PBS (supplemented with 0.1% Bovine Serum Albumine (BSA) and 0.01% NaN_3) were mixed with an appropriate volume of moAb solution (prechilled to the 4°C). The cells were incubated for 45 min in an ice bath, and subsequently washed twice with 500 μl of PBS (supplemented as above). The FITC-conjugated goat anti-mouse secondary antibody solution was added to the washed cell pellet for another 45 min. During this step and further on, the tubes with cells were protected from light. After the incubation time, the cells were washed and resuspended in 500 μl of diluent. Unlabeled IgG1 for CD11b and IgG2a for CD14 were used as a negative control in the first incubation. To determine the expression of the lactoferrin receptor on the cell surface a different procedure was used. 2.5×10^5 HL60 cells in 50 μl of PBS (supplemented as above) were incubated for 45 min in an ice bath in the presence of 10 μg/ml of lactoferrin. Then the cells were washed twice, and rabbit anti-lac-

toferrin antibody was added for a subsequent 45 min incubation. After the next two washings, the cells were incubated with an FITC-conjugated goat anti-rabbit antibody. When the last incubation terminated, the cells were washed and resuspended in 500 μl of diluent. In this case, a normal rabbit Ig fraction was used as a negative control in the second incubation. Cell surface fluorescence was measured using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Damaged cells were labelled by adding 5 μl of Propidium Iodide solution (25 μl/ml) to each test tube just before data acquisition. Data for damaged cells were not analyzed. Data analysis was performed using a Becton Dickinson Cell Quest software.

EC_{50} (effective concentration 50%)

EC_{50} is the concentration of a compound required to achieve differentiation of 50% of the cells exposed to the tested compound. EC_{50} was read out from the graph delineated for each experiment, and mean EC_{50} was calculated from at least 3 experiments.

Inhibition of proliferation

The cells (10^5 cells/well for 72 h of exposure, 1×10^3 HL60 and d_2c cells/well and 2×10^3 of normal human fibroblasts and keratinocytes for 144 h of exposure) were seeded on 96-well culture plates (Flat-Bottom/Low Evaporation Lid, Costar Cambridge, MA) in 75 μl of an appropriate medium. After 24 h, 75 μl aliquots of the medium containing the desired volumes of tested compound solutions were added to particular wells. Control wells were filled with a medium without any compound. During the following either 72 or 144 h, the plates were incubated under standard tissue culture conditions. For the last 3–4 hours of incubation 20 μl of MTT solution were added to each well (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, St. Louis, MO); stock solution: 5 mg/ml). In their mitochondria the viable cells reduce a pale yellow MTT to a navy blue formazan, so the more viable cells are contained in the well, the more MTT will be reduced to formazan. When incubation time had been completed, 80 μl of the lysing mixture were added to each well (lysing mixture: 225 ml dimethylformamide, 67.5 g sodium dodecyl sulphate (both from Sigma, St. Louis, MO) and 275 ml of distilled water). After 24 h, when formazan crystals had been dissolved, the optical densities of the samples were read on an Uniskan II photometer (Labsystems) at a 570 nm wavelength.

Each experimental sample was at least in triplicate and each experiment was performed at least three times. The result for a single probe was presented as a percent of the control (cells grown on the same plate in the same culture medium, but without the tested compound). Mean cell viability for all experiments was counted and presented on graphs with standard deviation. Ethanol which was used as a solvent (in dilution corresponding to its highest content applied to the tested compounds) did not exert considerable effect on cell proliferation ($p < 0.05$).

Table 1. Differentiation of HL60 cells induced by 1,25-dihydroxyvitamin D_3 and its analogues

Compound	Concn. (M)	Phagocytosis mean (% ± SD)	NBT mean (% ± SD)
Control	none	1 ± 1	3 ± 3
1,25-$(OH)_2$ D_3	10^{-6}	89 ± 1*	95 ± 2*
	10^{-7}	76 ± 1*	89 ± 3*
	10^{-8}	54 ± 3*	65 ± 6*
	10^{-9}	23 ± 1*	26 ± 5*
Analogue 1	10^{-6}	84 ± 4*	90 ± 6*
	10^{-7}	77 ± 6*	80 ± 3*
	10^{-8}	50 ± 7*	49 ± 10*
	10^{-9}	17 ± 13	15 ± 12
Analogue 2	10^{-6}	88 ± 4*	93 ± 1*
	10^{-7}	76 ± 10*	82 ± 9*
	10^{-8}	49 ± 13*	51 ± 6*
	10^{-9}	6 ± 4	7 ± 3
Analogue 3	10^{-6}	84 ± 2*	89 ± 3*
	10^{-7}	12 ± 3*	8 ± 5
	10^{-8}	2 ± 2	4 ± 3
	10^{-9}	2 ± 3	5 ± 2
Analogue 4	10^{-6}	63 ± 8*	70 ± 4*
	10^{-7}	8 ± 6	10 ± 6
	10^{-8}	2 ± 2	2 ± 2
	10^{-9}	1 ± 2	1 ± 1

HL60 cells were cultured with either 1,25-dihydroxyvitamin D_3 or its analogues for 96 h. The table presents results of at least three experiments. Results are presented as percent of total cells counted. Results marked with asterisk differ significantly from control ($p < 0.01$).

Statistical evaluation

We made use of Student's t-test for independent samples.

RESULTS AND DISCUSSION

Two functional assays were carried out to determine the effect of 1,25-dihydroxyvitamin D_3 analogues on cell differentiation and to compare it with that of 1,25-dihydroxyvitamin D_3. The results are listed in Table 1 (the concentrations of the analogues

Table 2. EC_{50} values (nM) for all compounds tested in phagocytosis and NBT reduction assays

Compound	Phagocytosis mean EC_{50}±SD (nM)	NBT mean EC_{50}±SD (nM)
1,25-$(OH)_2D_3$	7 ± 1	10 ± 2
Analogue 1	10 ± 2	10 ± 2
Analogue 2	12 ± 4	11 ± 2
Analogue 3	339 ± 1*	564 ± 9*
Analogue 4	610 ± 22*	475 ± 10*

The concentrations were read from at least three graphs and mean EC_{50} values were calculated. Results marked with asterisk differ significantly from results for 1,25-$(OH)_2D_3$ ($p < 0.01$).

tested were 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M). The activity of analogues **1** to **4** was found to be dose-dependent, and we recorded some differences between particular compounds. Analogues **1** and **2** revealed a differentiation inducing effect comparable with that of 1,25-dihydroxyvitamin D_3, whereas analogues **3** and **4** appeared to be less effective. The differences between the agents are described by the

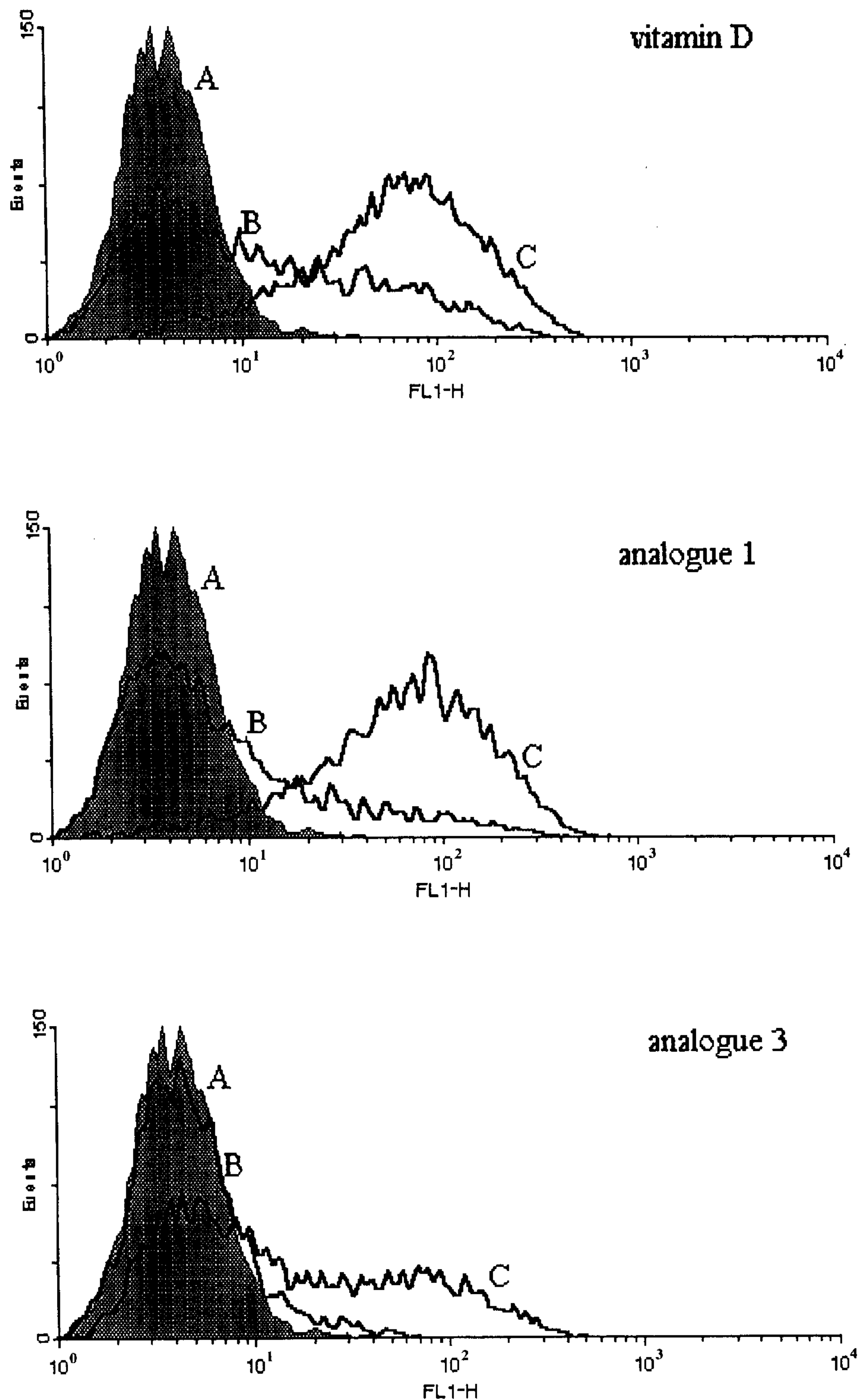

Fig. 2. The expression of CD14 antigen on the HL60 cells after 96 h of exposure either to 1,25-dihydroxyvitamin D_3, analogue 1 or analogue 3. The comparison with untreated cells: (A) untreated cells, (B) 10^{-10} M of compound, (C) 10^{-6} M of compound.

EC_{50} values calculated for particular compounds (see Table 2).

The determined antigen expression after a 4-day exposure of HL60 cells to 1,25-dihydroxyvitamin D_3 and its novel analogues confirmed that the cell differentiating activity of analogues **1** and **2** was comparable with that of 1,25-dihydroxyvitamin D_3. The increase in the expression of the cell-surface markers characteristic of monocytes was dose-dependent [19], as it can be seen in the graphs from flow cytometry in Fig. 2. For the purpose of illustration, the figure shows expression of CD14 after exposure of the cells to one of the more active (**1**) and to one of the less active (**3**) compounds, as well as to 1,25-dihydroxyvitamin D_3. Table 3 summarizes the results obtained in cytometric experiments. Part of the population of untreated HL60 cells express CD11b and CD14 cell-surface markers and the receptor for lactoferrin, but in low density (density is described by mean channel of fluorescence). The percentage of positive cells increased for all concentrations tested (10^{-6}–10^{-10} M), when differentiation was triggered by either 1,25-dihydroxyvitamin D_3 or analogue **1**. When differentiation was induced by analogue **2**, positive cell percentage increased only for the two higher concentrations (10^{-6} and 10^{-8} M). With analogue **3** and analogue **4** as differentiation triggering factors, the percentage of positive cells increased only for the highest concentration (10^{-6} M). As shown by data of Table 3, for 1,25-dihydroxyvitamin D_3 the concentration 10^{-10} M was still sufficient to cause an increase either in percentage or in density of the expression of all antigens studied (all six results are significantly higher than those for the control). For analogue **1** four out of six results and for analogue **2** one out of six results are significantly higher than for the control (at the concentration of 10^{-10} M). For analogue **3** and analogue **4**, the results are comparable with those of the control at concentrations 10^{-8} M and 10^{-10} M.

We also studied the proliferation of the cells exposed either to 1,25-dihydroxyvitamin D_3 or its analogues. HL60 cells, normal human keratinocytes, normal human skin fibroblasts and human keratinocytes transfected with human papillomavirus type 16 (d_2c cell line) were exposed to the compounds tested, at doses ranging from 10^{-9} (fibroblasts from 10^{-12}) to 10^{-6} M. An exposure of 72 h was not long enough to produce inhibition of cell growth (data not shown). When exposure time was extended to 144 h, we observed an inhibition of proliferation. There were distinct differences between the compounds and between the cells of various origin. The inhibition of cell proliferation after 144 h of exposure to the tested compounds is plotted in Fig. 3. Experimental data confirmed an earlier observation of ours — the activity of 1,25-dihydroxyvitamin D_3, analogue **1** and analogue **2**, was higher than that of analogue **3** and analogue **4** (in all the assays performed). The proliferation of HL60 cells and normal human fibroblasts was strongly affected by the 144 h-lasting exposure either to 1,25-dihydroxyvitamin D_3 or to its analogues. The proliferation of normal human keratinocytes and d_2c cells was only slightly affected by 1,25-dihydroxyvitamin D_3, analogue **1** and analogue **2**, but

Table 3. Expression of CD11b and CD14 cell-surface markers and lactoferrin (LF) receptor on HL60 cells cultured with either 1,25-dihydroxyvitamin D_3 or its analogues

Compound	Concn. (M)	CD11b		CD14		LF receptor	
		% ± SD	MC ± SD	% ± SD	MC ± SD	% ± SD	MC ± SD
Control	none	47 ± 10	7 ± 1	27 ± 15	6 ± 2	27 ± 15	8 ± 0
1,25-$(OH)_2$	10^{-6}	96 ± 4*	51 ± 20**	90 ± 5*	91 ± 27*	72 ± 6*	10 ± 0*
D_3	10^{-8}	90 ± 5*	38 ± 13**	83 ± 7*	94 ± 23*	74 ± 7*	11 ± 2**
	10^{-10}	76 ± 7**	22 ± 5*	56 ± 4**	48 ± 0*	58 ± 25	10 ± 2
Analogue 1	10^{-6}	97 ± 3*	52 ± 20**	87 ± 10*	93 ± 37**	75 ± 3*	10 ± 0*
	10^{-8}	87 ± 3*	39 ± 12*	84 ± 9*	89 ± 34**	72 ± 13**	11 ± 1*
	10^{-10}	65 ± 4**	15 ± 0*	44 ± 10	41 ± 11*	45 ± 18	8 ± 0**
Analogue 2	10^{-6}	97 ± 3*	54 ± 22**	82 ± 13*	68 ± 26**	76 ± 2*	10 ± 0*
	10^{-8}	88 ± 5*	39 ± 18**	87 ± 6*	108 ± 32*	72 ± 7*	10 ± 1**
	10^{-10}	56 ± 35	10 ± 3	46 ± 34	14 ± 2**	39 ± 29	8 ± 1
Analogue 3	10^{-6}	84 ± 7*	29 ± 3*	63 ± 9**	56 ± 7*	61 ± 12**	9 ± 1**
	10^{-8}	42 ± 6	7 ± 1	45 ± 5	13 ± 4	31 ± 18	8 ± 0
	10^{-10}	41 ± 22	8 ± 2	31 ± 18	8 ± 3	27 ± 8	7 ± 0
Analogue 4	10^{-6}	94 ± 6*	40 ± 8*	83 ± 6*	74 ± 31**	57 ± 30	8 ± 2
	10^{-8}	48 ± 7	8 ± 2	41 ± 2	9 ± 0	44 ± 20	8 ± 1
	10^{-10}	41 ± 27	7 ± 2	42 ± 2	8 ± 0	28 ± 15	7 ± 1

HL60 cells were exposed to either 1,25-dihydroxyvitamin D_3 or its analogues for 96 h. Results of three experiments are presented as percent of positive cells (%) and mean channel (MC) of fluorescence of positive cells population (±SD). Percent of positive cells was calculated by subtraction of a common area under graphs (graph representing negative control and graph representing an expression of antigen) from an area under graph representing the expression of antigen. Results marked with asterisks differ significantly from control (*$p < 0.01$ and **$p < 0.05$).

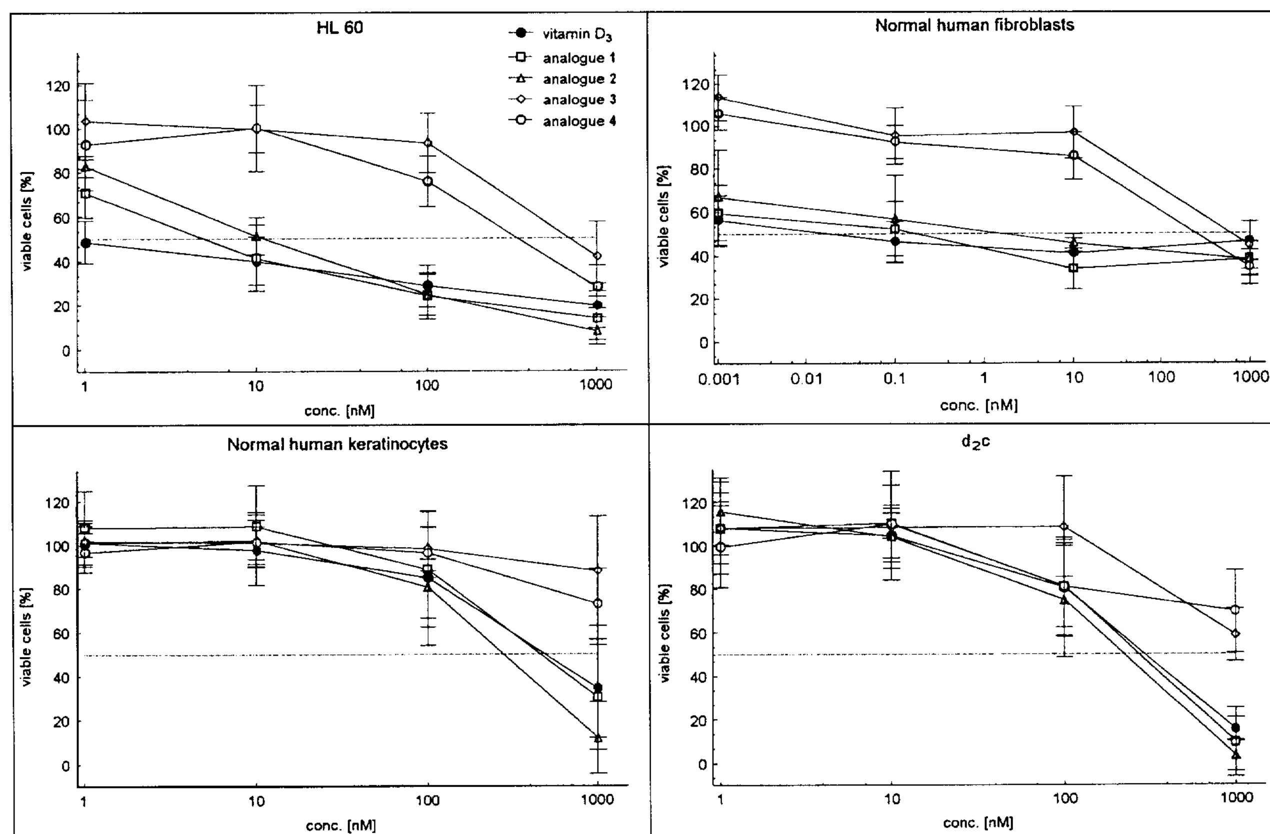

Fig. 3. The influence of either 1,25-dihydroxyvitamin D_3 or its analogues on proliferation of cells of various origin. Results are expressed as a mean percentage of viable cells $\pm$ SD (control samples considered as 100%) after 144 h of exposure to the compounds studied.

remained almost unaffected when exposed to analogue **3** and analogue **4**.

Analogues **1** and **2** can be considered as good candidates for further study of their contribution to cell differentiation *in vitro* in various cell line models. The way they affect cell differentiation and inhibit cell proliferation is similar to that of 1,25-dihydroxyvitamin D_3. The influence of all compounds tested on cell proliferation cannot be regarded as cytotoxic, since 72 h exposure was found to be insufficient to induce any change. An exposure as long as 144 h was needed to achieve growth inhibition, but the mechanism of this effect [6] is not apparent and should be a subject to further studies.

The presence of an additional hydroxyl in the side-chain of analogue **3** and analogue **4** did not improve their ability to stimulate cell differentiation. The shortening of the side-chain in the two analogues resulted in a substantial decrease in the biological activities studied. On the basis of the results obtained it is impossible to evaluate the contribution of the opposite chiralities at C-25 to the activity of analogues **3** and **4**. The synthesis of another series of side-chain extended and substituted analogues of 1,25-dihydroxyvitamin D_3 with the chiral center at C-25 is underway.

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