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Side-chain modified vitamin D analogs require activation of both PI 3-K and erk1,2 signal transduction pathways to induce differentiation of human promyelocytic leukemia cells

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Synthetic analogs of vitamin D for potential use in differentiation therapy should selectively regulate genes necessary for differentiation without inducing any perturbations in calcium homeostasis. PRI-1906, an analog of vitamin D_2, and PRI-2191, an analog of vitamin D_3 bind nuclear vitamin D receptor (nVDR) with substantially lower affinity than 1,25-dihydroxyvitamin D_3 (1,25-D_3), but have higher differentiation-inducing activity as estimated in HL-60 leukemia cell model. To examine how their increased differentiation-inducing activity is regulated we tested the hypothesis that membrane-mediated events, unrelated to nVDR, take part in the differentiation in response to PRI-1906 and PRI-2191. The induction of leukemia cell differentiation in response to the analogs of vitamin D was inhibited by LY294002 (phosphatidylinositol 3-kinase inhibitor), PD98059 (inhibitor of MEK1,2, an upstream regulator of extracellular-signal regulated kinase) and rapamycin ($p70^{S6K}$ inhibitor) pointing out that activation of signal transduction pathways unrelated to nVDR is necessary for differentiation. On the other hand, inhibition of cytosolic phospholipase A_2 accelerated the differentiation of HL-60 cells induced by either 1,25-D_3 or by the vitamin D analogs suggesting possible existence of a feedback loop between extracellular-signal regulated kinases and phospholipase A_2.

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Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; erk, extracellular-signal regulated kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; Me_2SO, dimethylsulfoxide; $NaCl/P_i$, phosphate-buffered saline; 1,25-D_3, 1,25-dihydroxyvitamin D_3; PI 3-K, phosphatidylinositol 3-kinase; VDR, vitamin D receptor.

The main function of 1,25-dihydroxyvitamin D_3 (1,25-D_3) is regulation of calcium homeostasis of the organism. This compound stimulates intestinal calcium absorption and bone calcium mobilization. Since 1,25-D_3 cooperates with parathyroid hormone in a precisely regulated manner it is now considered to be a hormone rather than a vitamin (Jones *et al.,* 1998). In the past two decades great progress has been made in understanding the role of 1,25-D_3 in differentiation of various cells, such as osteoclasts (Suda *et al.,* 1995), keratinocytes (Fogh & Kragballe, 2000) and monocytes (Abe *et al.,* 1981). It is presently well documented that it also induces differentiation of leukemia cells from patients and leukemia cell lines (Munker *et al.,* 1996).

The mechanism of action of 1,25-D_3 is rapidly being uncovered, however, it is still not fully understood. It is believed that in order to mediate its biological responses 1,25-D_3 must bind to a specific nuclear receptor protein, named nVDR (nuclear vitamin D receptor). Activated nVDR acts as a ligand-activated transcription factor and either upregulates or downregulates transcription of target genes (Jones *et al.,* 1998). However, during the past years experimental evidence for "nongenomic" signalling has been provided. It is well documented that 1,25-D_3 rapidly stimulates calcium transport in the perfused chick duodenum *in vivo* and formation of second messengers such as ceramides, inositols, cAMP and calcium and activation of a variety of protein kinases in the target cells *in vitro* (Norman *et al.,* 1999). It is generally accepted now that 1,25-D_3 generates its biological effects by these two mechanisms.

The existence of a separate VDR located in the cell membrane (mVDR) responsible for the "nongenomic" signalling was postulated many years ago (Nemere & Szego, 1981), but such receptor has not been cloned as yet. A putative mVDR was isolated from chick enterocytes and was further biochemically described (Nemere *et al.,* 1994; 1998; Pedrozo *et al.,* 1999; Nemere & Campbell, 2000), but the evidence that this particular receptor is responsible for all "nongenomic" signals is lacking. It should be noted that the physiological importance of the "nongenomic" signalling and its relation to nVDR-mediated signalling is not easy to elucidate. The open questions about membrane receptors are the same for all steroid hormones acting in animals (Pietras *et al.,* 2001).

As already mentioned, 1,25-D_3 induces differentiation of myeloid leukemia cells, such as HL-60, U937, THP-1 and M1. These cells, when incubated with 1,25-D_3, acquire the functional properties and express cell surface differentiation markers of monocytes (Brackman *et al.,* 1995; Oberg *et al.*, 1993; Schwende *et al.,* 1996; Abe *et al.,* 1981). Differentiation therapy has attracted many researchers, but undesirable side effects of natural 1,25-D_3 caused that its new analogs have been synthesized for this purpose. Analogs that could potentially be useful for differentiation therapy are deprived of the calcemic activity of 1,25-D_3, but have an even stronger differentiation-inducing potential.

A series of side-chain modified analogs was studied in the past with respect to their differentiation-inducing and antiproliferative activity. Our previous studies revealed that some analogs with extended side-chain are either comparably effective (Marcinkowska *et al.,* 1998a) or even more effective (Opolski *et al.,* 1999) than 1,25-D_3 in the induction of leukemia cell differentiation. Surprisingly, the effectiveness of a particular analog in the induction of cell differentiation is not related to its affinity for nVDR. Further studies revealed that 1,25-D_3 within minutes activates extracellular-signal regulated protein kinases (erk) 1 and 2 and induces their translocation to the nucleus in serum-starved HL-60 leukemia cells (Marcinkowska *et al.,* 1997). Pharmacological inhibition of the MEK/erk1,2 signal transduction pathway by PD98059, as well as inhibition of nuclear translocation by thapsigargin, reduce the cell-differentiating effects of 1,25-D_3 (Marcinkowska *et al.,* 1998b;

Marcinkowska, 2001). Another study revealed that also phosphatidylinositol 3-kinase (PI 3-K) is rapidly activated in human leukemia cells by 1,25-D_3 with the kinetics similar to that of erk1 and erk2 kinases (Hmama *et al.,* 1999). Inhibition of PI 3-K by either of the two specific inhibitors results in a concentration-dependent repression of the differentiation process. Similar effect of inhibition of 1,25-D_3-induced differentiation may be obtained with the use of an inhibitor of $p70^{S6K}$ protein kinase, namely with rapamycin (Marcinkowska *et al.,* 1998b). Possibly $p70^{S6K}$ is downstream of PI 3-K in the same signal transduction pathway, but further studies are necessary to reveal if this is the right explanation and to verify if $p70^{S6K}$ is actually rapidly activated by 1,25-D_3.

The erk1 and erk2 kinases that are activated by 1,25-D_3 belong to the larger family of mitogen-activated protein kinases (MAPKs). Different members of this protein kinase family are regulated by feedback loops in order to maintain precise levels of transcription factors activity, that eventually allow the cell to proliferate, differentiate or die. As was reported in a separate paper, inhibition of p38 kinase, another member of the MAPK family, potentiates the 1,25-D_3-induced differentiation through prolonged activation of erk1 and erk2 (Wang *et al.,* 2000). The data summarized above point out that "nongenomic" signalling is important for monocytic differentiation of leukemia cells.

For the studies presented in this paper we have selected side-chain modified analogs of vitamin D_2 (PRI-1906) and D_3 (PRI-2191) presented in Fig. 1, which are the most effective ones in inducing differentiation of the set studied by our group (Opolski *et al.,* 1999). These analogs are deprived of the calcemic activity of natural 1,25-D_3 (Chodyński *et al.,* 1997; Hansen *et al.,* 2000), possibly because of their lowered affinity for nVDR (Ikekawa & Ishizuka, 1992). It is therefore very probable that the increased differentiation-inducing activity of our analogs is mediated by mechanisms unrelated to nVDR. Thus, we wanted to find out if membrane-mediated activation of signal transduction pathways is necessary for the leukemia cell differentiation induced by PRI-1906 and by PRI-2191. Here we show that not only the 1,25-D_3-induced leukemia cell differentiation, but also differentiation induced by synthetic analogs of vitamin D requires activation of both the PI 3-K and erk1,2 signal transduction pathways. We also report that cytosolic phospholipase A_2 ($cPLA_2$) is involved in the 1,25-D_3-induced the differentiation and in the differentiation induced by vitamin D analogs, but in a negative manner. Differentiation was screened in our experiments by acquisition of the CD11b and CD14 cell surface markers (McCarthy *et al.,* 1983). CD11b

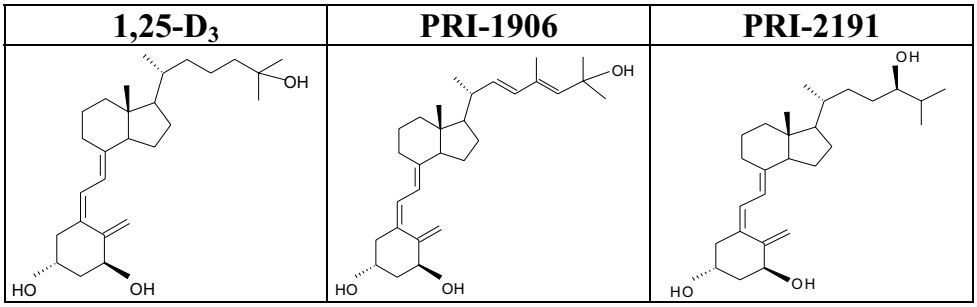

Figure 1. Chemical structures of the compounds studied.

The structures of 1,25-dihydroxyvitamin D_3 (1,25-D_3), vitamin D_2 analog (PRI-1906) and vitamin D_3 analog (PRI-2191).

is the α subunit of CR3 integrin that associates noncovalently with a β subunit partner, CD18. CD11b/CD18 is present on the surface of mature myeloid cells (Remold-O'Donnell, 1988). CD14 is another differentiation marker of monocytes. This protein is involved in responses to bacterial cell wall products (Wright *et al.,* 1990).

MATERIALS AND METHODS

Cell cultures. Human promyelocytic leukemia HL-60 cell line was obtained from the European Type Culture Collection. The cells were maintained in a suspension culture in RPMI1640 medium supplemented with 10% fetal calf serum (FCS, North American Origin; GIBCO BRL, European Division), 100 units/ml penicillin and 100 μg/ml streptomycin (both Polfa, Poland) and kept at standard cell culture conditions, i.e. humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Chemicals. 1,25-D_3 was obtained from the Pharmaceutical Research Institute (Warsaw, Poland). The analogs PRI-1906 and PRI-2191 were synthesized by the methods described earlier[1] (Chodyński *et al.,* 2000). The compounds were aliquoted and stored in glass ampoules under argon at –20°C. The amount of the compound in an ampoule was determined by UV spectrometry at 264 nm. Prior to use, the compound was dissolved in absolute ethanol to 100 μM and subsequently diluted in culture medium to the required concentration.

Protein G-Sepharose was from Pharmacia Biotech (Sweden); acrylamide, BIS and ammonium persulfate were from BioRad (Richmond, CA, U.S.A.); TEMED, myelin basic protein (MBP), sodium fluoride, sodium pyrophosphate, sodium orthovanadate, PMSF, sodium dodecyl sulfate, aprotinin, magnesium chloride, manganium chloride, Tris and EDTA were from Sigma (St. Louis, MO, U.S.A.).

Inhibitors. PD98059, LY294002 and $AACOCF_3$ were from Calbiochem (San Diego, CA, U.S.A.). Rapamycin was from Sigma (St. Louis, MO, U.S.A.). Inhibitors were dissolved in Me_2SO, aliquoted and kept at –20°C. Before experiments the inhibitors were thawed and diluted in culture medium to the required concentrations.

Antibodies. CD11b and CD14 monoclonal antibodies, mouse IgG1 and IgG2a fractions, all FITC conjugated, were from Sigma (St. Louis, MO, U.S.A.).

HL-60 differentiation assay. Cells were seeded in culture medium (supplemented with 10% FCS) on 24-well plates (Costar Cambridge, MA, U.S.A.) in a final volume of 1 ml. The cells were exposed for different times to 1,25-D_3, PRI-1906 or PRI-2191 alone or together with inhibitors. After completion of the exposure time the cells were collected by centrifugation (1000 r.p.m., 5 min), washed in phosphate-buffered saline ($NaCl/P_i$) and counted in a hemacytometer.

To determine CD11b or CD14 expression by flow cytometry, 2.5×10^5 HL-60 cells in 40 μl of $NaCl/P_i$ (supplemented with 0.1% BSA and 0.01% sodium azide) were mixed with 3 μl of monoclonal antibody solution (prechilled to 4°C). The cells were incubated for 45 min on ice, subsequently washed twice with 0.5 ml of $NaCl/P_i$ (supplemented as above) and resuspended in 0.5 ml of the diluent. The cells were protected from light. As negative controls FITC labeled IgG1 for CD11b and IgG2a for CD14 were used. The cell surface fluorescence was measured using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). The acquisition parameters were set for a negative control. Damaged cells were labeled by adding 5 μl of a propidium iodide solution (25 μg/ml) to each test tube just before

[1]Kutner A, Chodyński M, Szelejewski W, Odrzywolska M, Fitak H. (1999) Sposób otrzymywania pochodnych cholecalcyferolu oraz nowe związki pośrednie. Patent WO 9936400-A1.

the data aquisition. Data for damaged cells were not analysed. Data analysis was performed with the Cell Quest software (Becton Dickinson, San Jose, CA, U.S.A.). Figure 2 was prepared with the WinMDI 2.8 software (freeware by Joseph Trotter).

MAP kinase assay. HL-60 cells (3106 cells/sample, starved for 3 h in the absence of FCS) were exposed for the required time to either 0.1 μM or 1 μM 1,25-D_3 or PRI-1906. The cells were washed and lysed for 15 min on ice (lysis buffer: 50 mM sodium chloride, 10 mM Tris, 5 mM EDTA, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin and 1% Triton X-100, pH 7,4). Then the lysates were centrifuged at 14000 r.p.m. for 5 min. Supernatants were transferred to new tubes and 1.5 μg of anti-MAPK antibody was added. The lysates were incubated for 1 h under continuous shaking at 4°C. Then 40 μl of 50% protein G-Sepharose was added and incubated at 4°C for 2 h. The samples were centrifuged, washed twice with 1 ml of lysis buffer, once with 0.5 M sodium chloride and twice with 1 ml of kinase buffer (20 mM Tris, 10 mM magnesium chloride, 2 mM manganium chloride, 1 mM dithiothreitol, 1 mM EGTA, 100 μM sodium orthovanadate; pH 7.6). The pellets with immunoprecipitated erk1 and erk2 were soaked with 10 μl of kinase buffer. One μl of MBP solution (2 mg/ml) and 1 μCi of [γ-^{32}P]ATP were added to each tube and samples were kept at 30°C for 15 min. The reaction was stopped by adding 10 μl of 2 × SDS sample buffer and heating to 100°C for 5 min. The eluted proteins were separated by SDS/PAGE on a 14% resolving gel, fixed, dried and subjected to autoradiography, which was performed with an intensifying screen at –70°C with Kodak films.

Statistical evaluation. For statistical analysis experiments were repeated at least three times. Statistical analysis was performed with the Statistica software (StatSoft, Inc.). We used two-tailed Student's *t*-test for independent samples to assess the significance of differences between groups.

RESULTS

HL-60 cells may be exposed to 1,25-D_3 without inducing cytotoxicity for a very long time. 1,25-D_3-induced differentiation of HL-60 cells is concentration- and, up to some limit, time-dependent. Figure 2 shows how the expression of CD11b (A) and CD14 (B) in HL-60 cells changes with the time of exposure. HL-60 cells were exposed for two, four or six days either to 1,25-D_3 or to the most active analog in increasing concentrations. As presented earlier, the ethanol vehicle did not induce leukemia cell differentiation (Marcinkowska *et al.,* 1998a; Opolski *et al.,* 1999). Since a 48-h exposure of the cells to high concentrations of 1,25-D_3 or the side-chain modified analogs was sufficient to induce a significant increase in CD11b and CD14 and since some of the inhibitors used could be toxic after longer incubation time, all subsequent experiments were carried out under these conditions. On the other hand, the increased differentiation-inducing activity of PRI-1906 and PRI-2191 towards HL-60 cells could be seen after longer exposure and in the case of PRI-1906 is apparent even for very low concentrations (Opolski *et al.,* 1999).

Past studies revealed that 1,25-D_3 activates PI 3-K in human leukemia cells THP-1 (Hmama *et al.,* 1999). Another study demonstrated that pharmacological inhibition of PI 3-K in HL-60 cells reduces the 1,25-D_3-induced cell differentiation (Marcinkowska *et al.,* 1998b). Here we determined if the differentiation of leukemia cells induced by vitamin D side-chain modified analogs is also PI 3-K dependent. HL-60 cells were exposed for 48 h to either 1 μM 1,25-D_3, as a positive control, or to 1 μM PRI-1906 and 1 μM PRI-2191. Additional cell samples were exposed simultaneously to either 1,25-D_3 or the analogs and to an inhibitor of PI 3-K, LY294002. The concen-

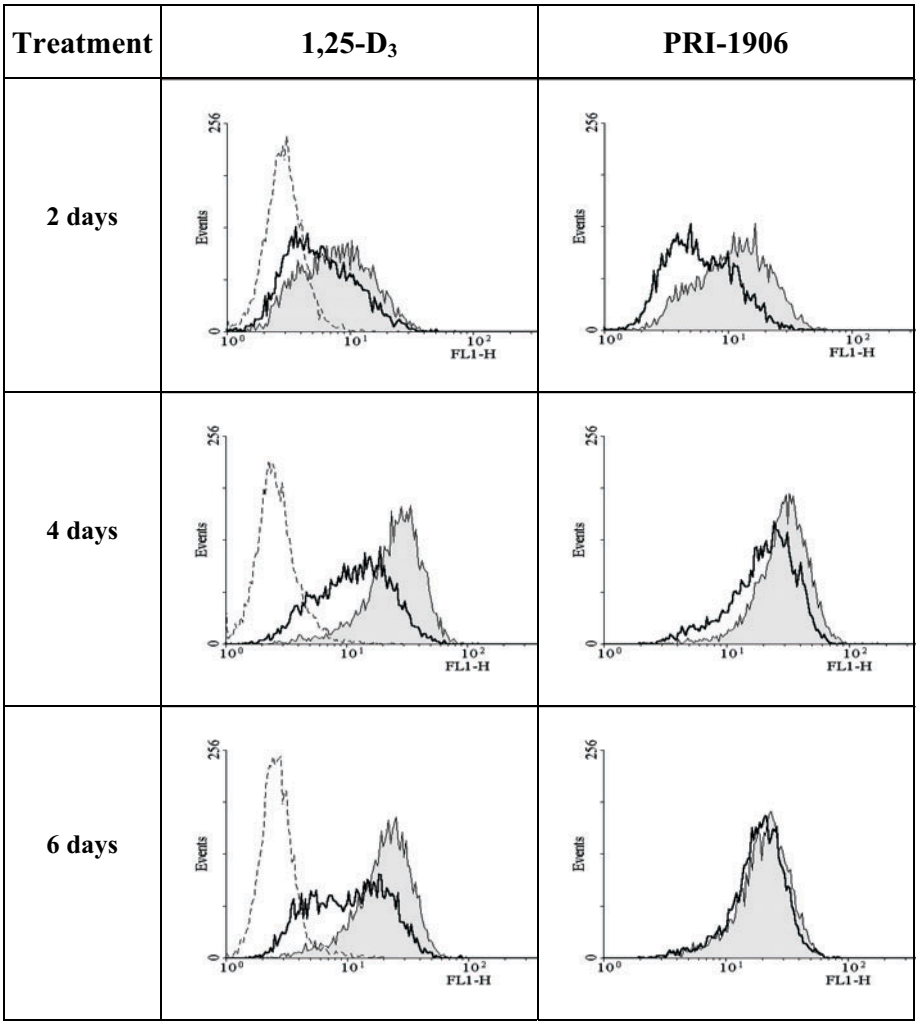

Figure 2A. Expression of differentiation cell-surface markers on HL-60 cells.

HL-60 cells were incubated in RPMI 1640 containing 10% FCS for the indicated times with either 1,25-D_3 or PRI-1906 in various concentrations. At the end of the incubation, expression of CD11b (**A**) and CD14 (**B**) was determined by flow cytometry. Cells were exposed to the compounds at the concentration of 1 μM (shadowed areas) and 0.1 nM (areas under thick black line). Graphs representing untreated cells (dashed line) are in the left column. Graphs were prepared with the WinMDI 2.8 freeware.

tration of LY294002 was adopted from the published papers (Hmama *et al.,* 1999; Marcinkowska *et al.,* 1998b). The Me_2SO vehicle alone did not inhibit HL-60 cell differentiation (Marcinkowska *et al.,* 1998b). As presented in Table 1, the differentiation of HL-60 promyelocytic leukemia cells induced by vitamin D side-chain modified analogs is PI 3-kinase dependent. The presence of LY294002 in the culture medium markedly inhibited the induced expression of CD11b and CD14 cell differentiation antigens. In all flow cytometry experiments the data were collected only for viable cells (propidium iodide non-incorporating). Separate experiments, revealed that simultaneous exposure of the cells to a differentiating agent and to the inhibitor did not cause high cytotoxicity. Cell cycle analysis performed with the Modfit software (Becton Dickinson) showed that the percentage of cells in sub-G_0 phase was below 5% in all samples (not shown).

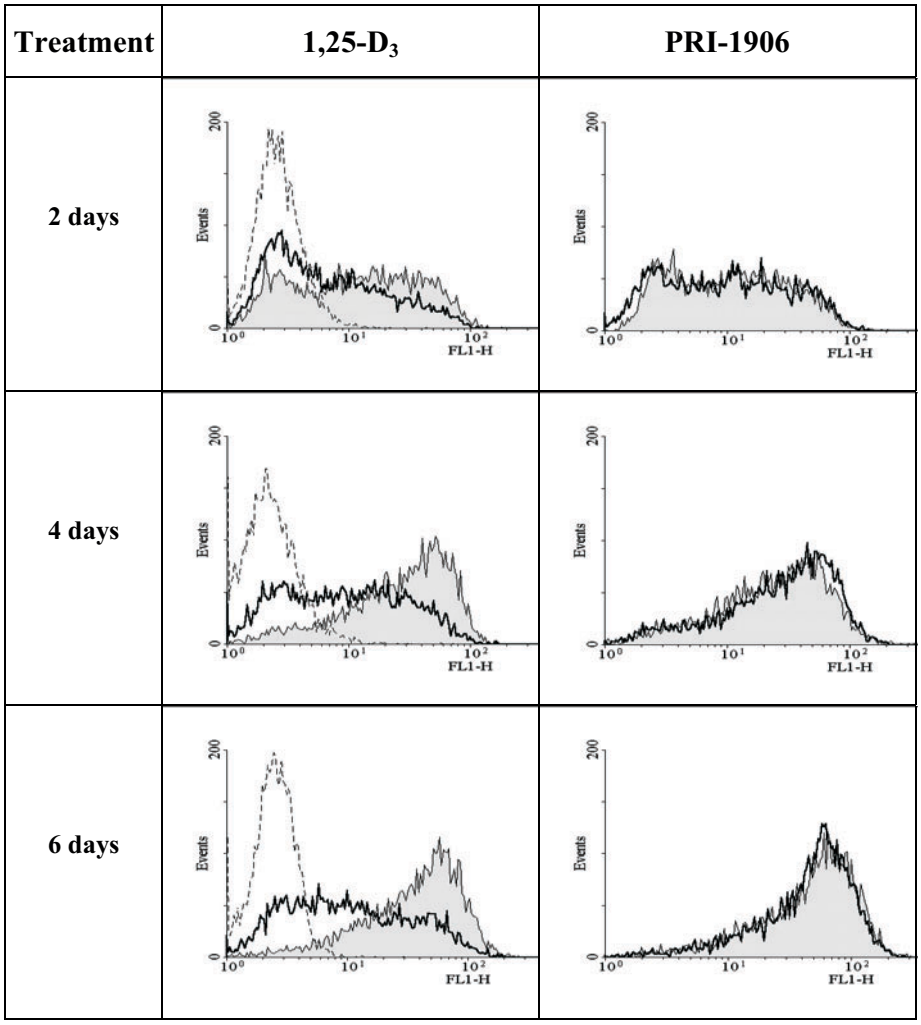

Figure 2B. Legend on the previous page.

P70^{S6K} protein kinase is a possible downstream element in the PI 3-K signal transduction pathway activated by various stimuli (Chung *et al.,* 1994). An involvement of p70^{S6K} in the 1,25-D_3-induced leukemia cell differentiation was suggested in the past (Marcinkowska *et al.,* 1998b). We investigated if also the differentiation induced by side-chain modified analogs of vitamin D may be inhibited with rapamycin, which blocks the activity of p70^{S6K}. The results obtained in this experiment and presented in Table 1 indicate that activation of p70^{S6K} is required for differentiation of leukemia cells induced by the vitamin D analogs studied here.

The erk1 and erk2 kinases are activated by 1,25-D_3 (0.1 μM to 1 μM) within minutes in serum-starved HL-60 cells (Fig. 3). We show here that also a side-chain modified analog of vitamin D_2 is able to induce rapid activation of these kinases. Serum starved HL-60 cells were stimulated for 10 min with either 1,25-D_3 or PRI-1906. Then, the activity of erk1 and erk2 was assayed by measuring the ability of immunoprecipitated kinases to phosphorylate myelin basic protein (MBP). Moreover 1,25-D_3-induced differentiation of HL-60 cells may be abolished by PD98059 (Marcinkowska, 2001), an inhibitor of MEK1 and MEK2, which are the only known and direct upstream

activators of erk1 and erk2 (Robinson & Cobb, 1997). The question we examined next was whether the differentiation of leukemia cells transduction pathway and regulates PKC by a feedback loop. To asses whether $cPLA_2$ is involved in the 1,25-D_3-induced differentiation

Table 1. Effects of LY294002, PD98059 and rapamycin on monocytic differentiation of HL-60 cells

Cell-surface marker	CD11b		CD14	
Treatment	[MC±S.D.]	[%±S.D.]	[MC±S.D.]	[%±S.D.]
untreated	3±0.3	2±1.2	2.8±0.2	4.4±1.3
1 μM 1,25-D_3	18.9±2.2	95±1.9	15.1±2.4	51.8±3.8
1 μM 1,25-D_3 + 20 μM LY294002	9.6±2.2**	70.9±15.7*	3.5±0.8**	8.4±3.3**
1 μM 1,25-D_3 + 40 μM PD98059	9±1.9**	70.2±14.6*	5.1±0.6**	24.6±7.1**
1 μM 1,25-D_3 + 1 μM rapamycin	10.8±1.8**	82.1±7.7*	4.4±0.6**	14±2.5**
1 μM PRI-1906	20±2.6	96.8±1.1	12.8±0.8	51.5±2
1 μM PRI-1906 + 20 μM LY294002	10±2.6**	70.1±13.5*	3±0.2**	4.9±0.9**
1 μM PRI-1906 + 40 μM PD98059	9.3±2**	70.5±14.3*	4.9±0.1**	21.9±1.4**
1 μM PRI-1906 + 1 μM rapamycin	10.8±1.8**	77.1±11.9*	4.1±0.5**	12.1±2.2**
1 μM PRI-2191	18.7±3.1	95.2±2	12.6±1.6	49.9±5.1
1 μM PRI-2191 + 20 μM LY294002	10.3±2.6*	72.8±13.1*	3±0.3**	5.1±1.3**
1 μM PRI-2191 + 40 μM PD98059	9.3±1.7**	72.7±11.6*	6.4±0.4**	22.6±4.5**
1 μM PRI-2191 + 1 μM rapamycin	10.1±1.6**	76.3±8.6**	4.1±0.5**	11.9±2.1**

HL-60 cells were exposed for 48 h to differentiation inducing factor alone (1 μM 1,25-dihydroxyvitamin D_3 (1,25-D_3), 1 μM PRI-1906 or 1 μM PRI-2191) or together with an inhibitor (20 μM LY 294002 inhibitor of PI3-K, 40 μM PD 98059 inhibitor of MEK or 1 μM rapamycin inhibitor of $p70^{S6K}$). Then expression of CD11b and CD14 on the surface of viable cells from particular samples was determined by flow cytometry. MC (mean channel) of fluorescence was calculated using CellQuest software. Positive cells [%] fall into channels of fluorescence higher than the fluorescence of irrelevant isotype-matched IgG. Results marked by asterisks differ significantly from the results obtained for samples treated with the respective differentiation inducing factor alone (*$P < 0.05$; **$P < 0.01$).

induced by the vitamin D side-chain modified analogs is sensitive to PD98059. Concentration of PD98059 was adopted from previous experiments (Marcinkowska, 2001). The data presented in Table 1 show that expression of CD11b, and CD14 may be significantly inhibited by PD98059 in cells differentiated by the vitamin D side-chain modified analogs.

The involvement of $cPLA_2$ in the 1,25-D_3-induced differentiation was reported in the past, but was restricted to the differentiation of growth zone chondrocytes (Schwartz *et al.,* 1988). In growth zone chondrocytes, regulation of PLA_2 is involved in the activation of protein kinase C (PKC), but it is still unclear if PLA_2 is an upstream positive regulator of PKC, or is downstream of PKC in the signal

of promyelocytic leukemia cells, HL-60 cells were simultaneously exposed for 24 h to 1,25-D_3 and to an inhibitor of PLA_2. The inhibitor $AACOCF_3$ targets only the cytosolic form of PLA_2 (Street *et al.,* 1993). The concentration of $AACOCF_3$ was the same as previously used in experiments with chondrocytes (Helm *et al.,* 1996). Surprisingly, the results of these experiments showed that $cPLA_2$ is involved in the 1,25-D_3-induced differentiation of leukemia cells, but in a negative manner. As presented in Fig. 4 the inhibitor itself does not induce differentiation of HL-60 cells, but in concert with 1,25-D_3 it potentiates the acquisition of the CD11b differentiation marker. To investigate if this is also the case for the differentiation induced by side-chain modified analogs of

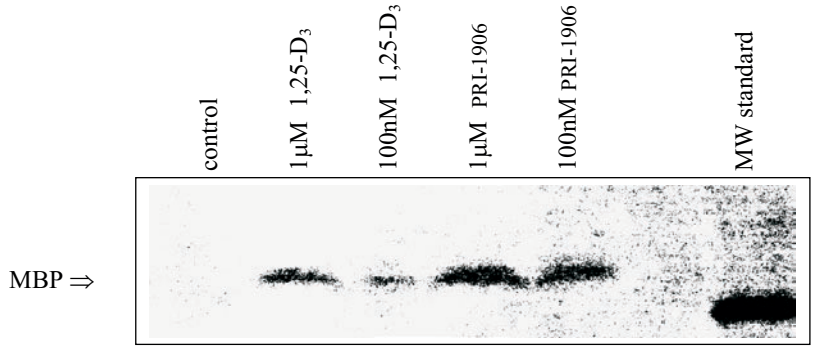

Figure 3. Activation of erk1 and erk2 in response to 1,25-D_3 or PRI-1906.

Serum starved HL-60 cells were treated for 10 min with 1,25-D_3 or PRI-1906 at two concentrations. Control cells were left untreated. Activation of erk1 and erk2 was assayed by their ability to phosphorylate meylin basic protein (MBP).

vitamin D, HL-60 cells were cultured with either PRI-1906 or PRI-2191 and $AACOCF_3$ at the same time. In these experiments 1,25-D_3 was included as a positive control. As shown in Table 2 the presence of $AACOCF_3$ in culture medium significantly increased the expression of CD11b, but not of CD14.

DISCUSSION

The design of new vitamin D analogs for potential use in differentiation therapy should lead to the synthesis of analogs that could selectively regulate genes necessary for differentiation without inducing any perturbations in calcium homeostasis. Since the mechanism of action of 1,25-D_3 is not fully understood, the current approach in developing vitamin D analogs involves full biological testing of all newly designed compounds. According to Brown (2000) the potential mechanisms through which this selectivity could be achieved are: (1) altered systemic transport, (2) altered metabolism, (3) altered conformational change in nVDR, and finally (4) altered binding to mVDR. The three-dimensional structure of the ligand binding domain (LBD) of nVDR has only very recently become known from computational models (Yamada *et al.,* 2001) and from the crystal structure (Rochel *et al.,* 2000; Tocchini-Valentini *et al.,* 2001). This means that the design of the presently studied analogs was not based on their binding to nVDR LBD. Moreover, the crystal structures of nVDR complexed to 20-epi analogs of 1,25-D_3 revealed that LBD conformation is the same as for 1,25-D_3 (Tocchini-Valentini *et al.,* 2001). Although the increased differentiation-inducing activity of these 20-epi analogs was ascribed exclusively to the higher stability of the nVDR-agonist complex, augmented activation of the mVDR signalling should also be considered (Yan *et al.,* 2002).

The potential involvement of mVDR in the activity of analogs complicates the situation. mVDR has been isolated only from chick intestinal cells (Nemere *et al.,* 1994; Nemere & Campbell 2000) and rat osteoblastic cells (Nemere *et al.,* 1998; Pedrozo *et al.,* 1999). However, many studies have demonstrated the physiological importance of membrane-mediated effects for the 1,25-D_3-induced leukemia cell differentiation (Marcinkowska *et al.,* 1998b; Hmama *et al.,* 1999; Wang *et al.,* 2000; Marcinkowska, 2001; Yan *et al.,* 2002). The relation of the nVDR-mediated "genomic" signalling to the mVDR-mediated "nongenomic" signalling is still unclear. In our understanding a cross-talk between these two mechanisms is necessary for cell differentia-

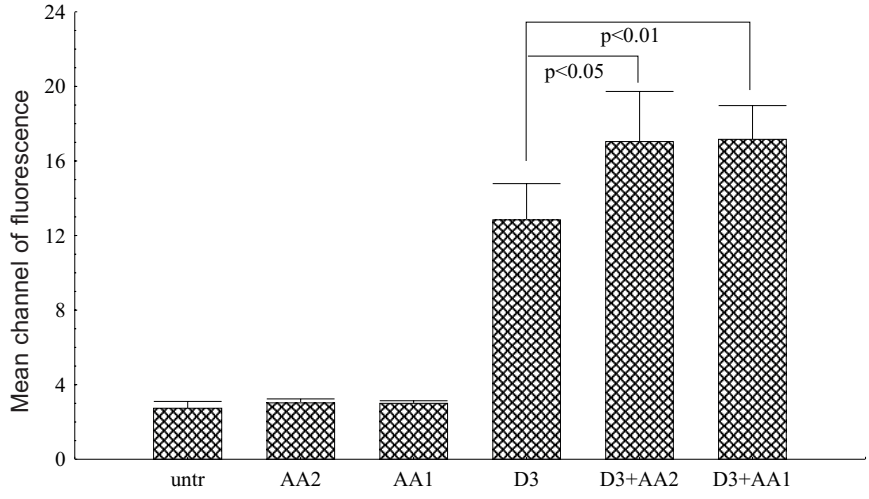

Figure 4. Potentiation of 1,25-D_3-induced differentiation by inhibitor of $cPLA_2$.

Expression of the CD11b cell-surface marker on HL-60 cells treated with either 1 μM 1,25-D_3 alone or together with $AACOCF_3$ for 24 h. Means ±S.D. are presented. Untr, untreated; AA2, 2 μM $AACOCF_3$; AA1, 1 μM $AACOCF_3$; D_3, 1 μM 1,25-D_3.

tion. One possibility to consider is that the transcriptional activity of liganded nVDR could be modulated by transcription factors either activated or repressed downstream of mVDR. A second possibility is that phosphorylation of nVDR, necessary for its activation, may be differentially regulated by membrane-mediated events.

Here we studied two active analogs of vitamin D_2 and vitamin D_3. Of a series of the previously obtained vitamin D analogs we selected for this study PRI-2191 as the most active analog of vitamin D_3, and PRI-1906 as the most active analog of vitamin D2. Since our vitamin D analogs are more effective than 1,25-D_3 in *in vitro* induction of leukemia cell differentiation, their increased activity could not be mediated by an altered systemic transport. Therefore we hypothesized that enhanced activation of "nongenomic" signalling could contribute to the biological effect of our analogs.

In this communication we show that activation of membrane-mediated effects are necessary for the completion of leukemia cell differentiation induced not only by natural 1,25-D_3,

Table 2. Effect of $cPLA_2$ inhibitor on monocytic differentiation of HL-60 cells

Cell-surface marker	CD11b		CD14	
Treatment	[MC±SD]	[%±SD]	[MC±SD]	[%±SD]
untreated	3±0.3	2±1.2	2.8±0.2	4.4±1.3
1 μM 1,25-D_3	18.9±2.2	95±1.9	15.1±2.4	51.8±3.8
1 μM 1,25-D_3 + 2 μM $AACOCF_3$	25.1±3.5**	99.3±0.5**	14.96±2.1	55.8±3.5
1 μM PRI-1906	20±2.6	96.8±1.1	12.8±0.8	51.5±2
1 μM PRI-1906 + 2 μM $AACOCF_3$	25.9±3.6*	99.5±0.2**	13.8±1.4	55.6±5.2
1 μM PRI-2191	18.7±3.1	95.2±2	12.6±1.6	49.9±5.1
1 μM PRI-2191 + 2 μM $AACOCF_3$	24.5±3.5*	99.1±0.5**	13.2±0.7	53.1±2.6

HL-60 cells were exposed for 48 h to a differentiation inducing factor alone (1 μM 1,25-dihydroxyvitamin D_3 (1,25-D_3), 1 μM PRI-1906 or 1 μM PRI-2191) or together with an inhibitor of phospholipase A_2 (2 μM $AACOCF_3$). Then expression of the CD11b and CD14 on surface of viable cells was determined by flow cytometry. MC (mean channel) of fluorescence was calculated using CellQuest software. Positive cells [%] fall into channels of fluorescence higher than the fluorescence of irrelevant isotype-matched IgG. Results marked by asterisks differ significantly from the results obtained for samples treated with respective the differentiation inducing factor alone (*$P < 0.05$; **$P < 0.01$).

but also by synthetic analogs with lowered affinity for nVDR, as long as they retain the cell differentiating activity equivalent to or higher than that of 1,25-D_3. We report that activation of the PI 3-K and the MEK/erk1,2 signal transduction pathway is necessary for the process of differentiation induced by vitamin D analogs. From our earlier studies we could deduce that these two pathways are separate (Marcinkowska *et al.,* 1998b). The activation of $p70^{S6K}$ is also necessary, but it is unclear if this is a direct membrane-mediated effect of vitamin D analogs, since evidence that 1,25-D_3 rapidly activates this kinase is still lacking. Here we show that also $cPLA_2$ is involved in the regulation of leukemia cell differentiation induced by either 1,25-D_3 or by the side-chain modified analogs. The nature of this regulation is purely a matter of speculation now. However, it is well documented that $cPLA_2$ may be upstream regulated by erk1 and erk2 (Geijsen *et al.,* 2000). On the other hand, 1,25-D_3 activates erk1 and erk2, which in turn could lead to activation of $cPLA_2$. Inhibition of $cPLA_2$ may cause disruption of a feedback loop and overactivation of erk1 and erk2, leading to increased differentiation of HL-60 cells. The same mechanism as for 1,25-D_3 is involved in the differentiation induced by side-chain modified analogs of vitamin D. A hypothetical mechanism of cellular signalling activated in HL-60 cells during differentiation induced either by 1,25-D_3 or by its analogs is presented in Fig. 5.

There are still many open questions concerning the complex process of cell differentiation.

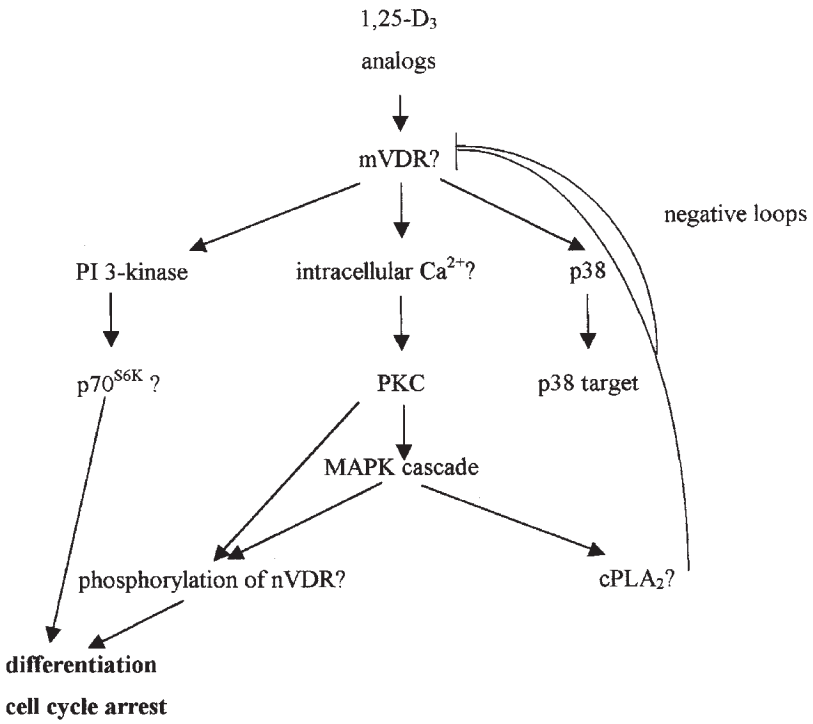

Figure 5. Hypothesis for the mechanism of differentiation induced by 1,25-D_3 or its analogs in HL-60 cells.

Activation of the putative membrane vitamin D receptor leads to the rapid activation of PI-3 kinase and its downstream effector $p70^{S6K}$, which is necessary for differentiation (Hmama *et al.,* 1999; Marcinkowska *et al.,* 1998b). It also activates MAPK cascades. PKC mediated activation of erk1 and erk2 leads to their translocation to the cell nucleus (Marcinkowska *et al.,* 1997) and is necessary for differentiation (Marcinkowska, 2001; Wang & Studzinski, 2001). Activation of p38, by a negative loop, downregulates 1,25-D_3-induced activation of erk1 and erk2 (Wang *et al.,* 2000). Another negative loop could possibly exist between products of PLA_2 and an upstream regulator of erk1 and erk2.

Although the inhibition of cell proliferation is a long term effect accompanying cell differentiation, Wang & Studzinski (2001) in their detailed study presented data supporting the hypothesis that differentiation could be divided into two phases in respect to cell proliferation. In the first phase, which is defined by activation of erk1 and erk2, the cells continue normal cell cycle. In the second phase, the cell cycle is blocked in G_1 phase and the activity of erk1 and erk2 is suppressed. It should be noted that increased expression of the CD11b and CD14 monocytic differentiation markers can be detected in both phases of cell differentiation process. It is therefore possible that activation of "nongenomic" signals is necessary for completion of the first, proliferative phase of differentiation.

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