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Structure–function analysis of vitamin D_2 analogs as potential inducers of leukemia differentiation and inhibitors of prostate cancer proliferation

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ABSTRACT

We characterized a structure–function relationships of four analogs of vitamin D_2 with extended and branched side-chains. We tested their ability to induce differentiation of human acute myeloid leukemia (AML) cells both *in vitro* and *ex vivo*. Our experiments on five human cell lines revealed substantial differences among tested analogs. Analogs with side-chains extended by one (PRI-1906) or two carbon units (PRI-1907) displayed similar or elevated cell-differentiating activity in comparison to 1,25-dihydroxyvitamin D_3 (1,25D), whereas further extending side-chain resulted in substantially lower biological activity (PRI-1908 and PRI-1909). Similar pattern of cell-differentiating activities to that observed in human cell lines has also been shown in blast cells isolated from patients diagnosed with AML. The ability of the analogs to activate expression of CYP24A1 gene has been studied in HL60 cell line. The analog PRI-1906 activated expression of CYP24A1 similarly to 1,25D, while PRI-1907 weaker than 1,25D. In addition, the analogs PRI-1906 and PRI-1907 were able to moderately inhibit proliferation and significantly activate expression of CYP24A1 mRNA in prostate cancer cells PC-3. Finally, we examined the molecular actions triggered by these analogs and found that their biological activity was related to their ability to induce expression and nuclear translocation of VDR and C/EBPβ.

1. Introduction

It is well documented that 1,25-dihydroxyvitamin D_3 (1,25D) is not only a potent anti-rachitic agent [1], but it is also a compound important for cell proliferation [2], cell differentiation [3] and immunomodulation [4]. Since differentiation of prostate cancer, breast cancer and myeloid leukemia cells may have beneficial therapeutic effects in pathological conditions [5], many vitamin D analogs with improved anti-proliferative and pro-differentiating activities, as well as lower calcemic effects have been designed. Even though some of the analogs are used for the treatment of patients with psoriasis [6], the mechanism of their selective activity is not known. The studies on the mechanism underlying biological effects of 1,25D analogs provide important information that allow to determine what structural modifications of 1,25D molecule are responsible for their changed biological properties.

It is well known that 1,25D binds to the vitamin D receptor (VDR), which heterodimerizes with the liganded retinoid X receptor (RXR), and binds to vitamin D response elements (VDREs) in the promoter regions of target genes [7]. Many of VDR target genes are connected with the calcium/phosphate homeostasis, but also with anti-proliferative and pro-differentiating actions of 1,25D in non-calcemic tissues. The gene which is the most strongly regulated by VDR is CYP24A1, encoding an enzyme responsible for degradation of 1,25D. This provides a feedback loop, which turns off the activity of 1,25D. In addition to triggering genomic responses described above, VDR is thought to mediate rapid non-genomic actions in target cells [8]. These actions include activation of multiple MAP kinase cascades [9–11] and PI3 kinase pathway [12,13]. Therefore an interplay between differentially activated genomic and non-genomic pathways could be a plausible explanation for selective activities of various analogs, however other possible reasons can be found in scientific literature. One of these is differential interaction of particular analogs with aminoacids that create VDR's ligand binding domain (LBD), and therefore different dissociations of given analog from VDR. It is thought that the differences in positioning helix 12 of LBD,

Abbreviations: AML, acute myeloid leukemia; 1,25D, 1,25-dihydroxyvitamin D_3; C/EBPβ, CCAAT/enhancer-binding protein beta; CYP24A1, 25-hydroxyvitamin D_3 24-hydroxylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Hsp90, heat shock protein 90; LBD, ligand binding domain; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

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and therefore changed interaction with co-activators may explain, why some analogs induce stronger transcriptional responses than 1,25D [14].

In our previous research we have described pro-differentiating activity of a side-chain modified analog of vitamin D_2, PRI-1906, which has lower calcemic potential than 1,25D [15–19]. In order to study a structure–function relationships, a series of vitamin D_2 analogs with extended and branched side-chains has been designed and their anti-proliferative activities were described [16,20]. In this paper we describe their pro-differentiating activities towards human myeloid leukemia cells from cell lines with various genetic lesions characteristic for acute myeloid leukemia (AML) and towards blasts from patients with AML. We examined their ability to increase expression of VDR and C/EBPβ transcription factors in leukemic cells. Then the expression of CYP24A1 mRNA induced by the analogs that exerted the strongest pro-differentiating action was measured. The ability of the analogs to inhibit proliferation and activate expression of CYP24A1 mRNA in prostate cancer cells has also been studied.

2. Materials and methods

2.1. Cell lines

HL60 cells were obtained from the European Collection of Cell Cultures. NB-4 cells were a kind gift from Prof. George P. Studzinski (University of Medicine and Dentistry of New Jersey), while PC-3 cells from Prof. Pirko Vihko (University of Helsinki). U-937, MV4-11 and MOLM-13 were purchased from German Resource Center for Biological Material (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cells were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). The cells were kept at standard cell culture conditions, i.e. humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cell number and viability were determined by hemocytometer counts and trypan blue (0.4%) exclusion. For all experiments the cells were suspended in fresh medium containing 1,25D, analog or the equivalent volume of ethanol as a vehicle control.

2.2. Isolation of mononuclear cells from patient's peripheral blood

Ten ml of peripheral blood was carefully layered onto the equal volume of LSM 1077 (PAA Laboratories GmbH, Pasching, Austria), and centrifuged at 400 × *g* for 30 min. The opaque interface containing the blast cells was transferred into fresh sterile tube, and washed three times with PBS. The cells at the density of 10^6 cells/ml were transferred to RPMI 1640 medium, supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin and grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The study was accepted by the local Ethical Committee. The patients who presented to the Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation, Wroclaw Medical University, were informed about the aim of the experiments and gave informed consent for this study.

2.3. Chemicals and antibodies

1,25D and all analogs were synthesized in the Pharmaceutical Research Institute (Warsaw, Poland). The compounds were aliquoted and stored in glass ampoules under argon at −20 °C. Amount of the analog in the ampoule was determined by UV spectrometry at 264 nm, compound was dissolved in an absolute ethanol to 100 μM, and subsequently diluted in the culture medium to the required concentration.

Antibodies CD14-FITC, CD14-PE, CD11b-FITC, CD3-PE and CD20-PE were from ImmunoTools (Friesoythe, Germany). Chemiluminescence Blotting Substrate was from Roche Diagnostics (Mannheim, Germany). Mouse monoclonal anti-VDR, anti-Hsp90, rabbit polyclonal anti-C/EBPβ and anti-Histone H1 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal anti-p42,44/Erk and anti-Histone H3 antibodies were from Cell Signalling Technology (Beverly, MA). Goat anti-rabbit IgG and anti-mouse IgG conjugated to peroxidase were from Jackson ImmunoResearch (West Grove, PA). Alamar Blue was purchased from Invitrogen (Carlsbad, CA). Other reagents were from Sigma (St. Louis, MO).

2.4. Determination of cell differentiation by flow cytometry

The expression of cell surface markers of monocytic differentiation was determined by flow cytometry. The cells were incubated with 1,25D or analogs and then stained with appropriate antibodies using protocols described below.

In case of human leukemic cell lines, following incubation with analogs, cells were washed twice in PBS and incubated with 1 μl of CD14-PE and 1 μl of CD11b-FITC for 1 h on ice. Next, they were washed three times with ice-cold PBS, fixed in 1.5% paraformaldehyde, and suspended in 0.5 ml PBS prior to analysis on FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

Blast cells were stained with different set of antibodies. Following incubation with CD14-FITC, CD3-PE, and CD20-PE antibodies (1 μl of each antibody per sample) on ice, blast cells were washed three times and suspended in 0.5 ml of PBS. To distinguish between viable and non-viable cells we used propidium iodide at final concentration of 0.25 μg/ml. During data analysis we excluded cells that emit fluorescence in the red channel (lymphocytes and non-viable cells).

The acquisition parameters were set for appropriate isotypic control. Data analysis was performed using WinMDI 2.9 software (freeware by Joseph Trotter).

2.5. Preparation of cell lysates

In order to prepare lysates, the cells (5×10^6) were washed 3 times with PBS and lysed for 20 min on ice in 80 μl of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100; pH 7.5) containing protease inhibitor cocktail (Roche Diagnostics). The lysates were separated by centrifugation for 5 min, at 18,000 × *g*, at 4 °C. Supernatants were designated the cytoplasmic (C) fraction, and the nuclei remaining in pellets after one washing were sonicated for 10 s in the same volume of lysis buffer as before (80 μl/5×10^6 cells). Following sonication, nuclei were centrifuged again for 5 min, at 18,000 × *g*, at 4 °C and the final supernatants were designated the nuclear (N) fraction. Samples were denatured by adding 20 μl of 5× SDS sample buffer and boiling for 5 min.

2.6. Western blotting

For western blotting, 25 μl of cell lysates (derived from 1.25×10^6 cells) was separated on 12% SDS-PAGE gels and transferred to the PVDF membranes. The membranes were then dried, and incubated sequentially with primary, and a horseradish peroxidase-conjugated secondary antibody (1 h, room temperature). The protein bands were visualized with a chemiluminescence. Then the membranes were stripped, dried again and probed with subsequent antibodies. Hsp90 was used as a control of equal loading and a quality of protein transfer. Scanned gels were quantified using ImageJ 1.34s software (freeware by Wayne Rasband, NIH).

2.7. cDNA synthesis and PCR

Total RNA was isolated using TriPure reagent (Roche Diagnostics) according to the manufacturer's recommendations. RNA quantity was determined using Nanodrop (Thermo Fisher Scientific Inc. Worcester, MA) and the quality of RNA was verified by gel electrophoresis. RNA was transcribed into cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Initially, CYP24A1 gene expression was assessed using semi-quantitative RT-PCR. In these experiments 32 PCR cycles were used to co-amplify CYP24A1 and 18S RNA endogenous control. Cyp24A1 primers were as follow: (fp) 5′-CCC ACT AGC ACC TCG TAC C-3′, and (rp) 5′-CGT AGC CTT CTT TGC GGT AG-3′. 18S rRNA primers and competimers (QuantumRNA™ 18S Internal Standards; Ambion, Austin, TX) were used in 1:6 ratio. Products of PCR reaction were separated in 1.5% agarose gels, stained with ethidium bromide and documented using GelDoc system (Bio-Rad, Hercules, CA). From the same cDNA samples Real Time PCR was performed using SYBR Green (A&A Biotechnology, Gdansk, Poland) in Applied Biosystems StepOne system. The sequence of CYP24A1 primers and reaction conditions were as described previously [19], while sequences of GAPDH were as follow: (fp): 5′-CAT GAG AAG TAT GAC AAC AGC CT-3′, (rp): 5′-AGT CCT TCC ACG ATA CCA AAG T-3′. Fold changes of mRNA levels in CYP24A1 gene relative to the GAPDH gene were calculated by relative quantification analysis.

2.8. Proliferation assay

PC-3 cells were seeded in 96-well culture plates in 100 μl of culture medium. After 24 h, next 100 μl of medium containing 1,25D, analogs or vehicle control was added to the respective wells. After next 96 h of incubation proliferation assay was carried out according to the manufacturer's instructions. Briefly, samples were incubated with Alamar Blue (20 μl/well) for 4 h. Then, fluorescence by excitation at 560 nm and emission at 590 nm was read on SpectraMAX Gemini XS Reader (Molecular Devices, Downington, PA).

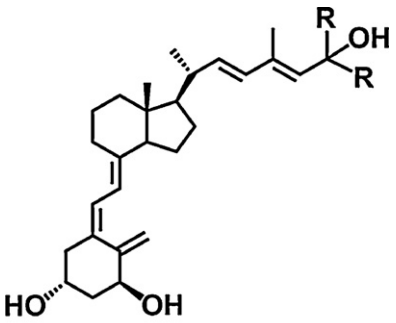
2.9. Statistical analysis

In order to analyze the results obtained in experiments with cell lines, Student's *t*-test for independent samples was used. Western blots were repeated 2 or 3 times, flow cytometry experiments were repeated 4 or 5 times, while PCR and proliferation assays were repeated 3 times.

3. Results

3.1. Vitamin D_2 analogs

The methods of synthesis of vitamin D_2 analogs tested in this study, as well as their anti-proliferative activities were presented before [15,16,20]. Previously, we showed that pro-differentiating properties of analogs are improved by two types of chemical modifications: introduction of an additional double bond in the side-chain of vitamin D_2 and extension of vitamin D side-chain by one or two [21] carbon units in an aliphatic and not alicyclic manner. In the series of analogs tested in present experiments we have combined both types of modifications. As shown in Fig. 1, the analogs differ one from another by the length of these aliphatic chains; PRI-1906, PRI-1907, PRI-1908 and PRI-1909 contain a pair of methyl, ethyl, n-propyl and n-butyl groups at C-25, respectively.



PRI-1906, R = -CH_3
PRI-1907, R = -CH_2CH_3
PRI-1908, R = -$CH_2CH_2CH_3$
PRI-1909, R = -$CH_2CH_2CH_2CH_3$

Fig. 1. The scheme of chemical structures of vitamin D_2 analogs used in the study. The analogs are modified in the side-chain, which was extended by one carbon and rigidified by two conjugated double bonds at C-22 and C-24. Two symmetrical aliphatic chains of different lengths were also introduced at C-25.

3.2. Differentiation-inducing activities of analogs towards HL60 cell line and blast cells from patients with AML

In order to study how changes introduced to the side-chains of vitamin D_2 analogs influence their pro-differentiating activities, HL60 cells were used. These cells which are frequently used in differentiation studies, were originally described as AML M3 [22], but further research has shown that they originate from subtype M2, according to FAB classification [23]. In the first screening 1,25D and analogs were used at 1 nM and 10 nM concentrations. The cells were exposed to the compounds for 96 h and then the expression of monocyte/macrophage differentiation markers CD11b and CD14 was studied in flow cytometry. Fig. 2A shows results for 1 nM analogs, while Fig. 2B for 10 nM analogs. These data show that the maximum pro-differentiating activity was achieved with PRI-1907 analog. This analog was significantly more active than 1,25D at both concentrations tested, and more active than PRI-1906 at 1 nM. The analogs with n-propyl (PRI-1908) and n-butyl (PRI-1909) aliphatic chains introduced at C-25 were less active than 1,25D. Interestingly, the pro-differentiating effects of PRI-1908 increased in dose-dependent manner, whereas PRI-1909 yielded same very low biological response at both 1 and 10 nM concentrations.

The four analogs were studied in experiments performed on blast cells isolated from patients with freshly diagnosed AMLs, who presented to the Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation, Wroclaw Medical University, in the first half of year 2010. Patient's characteristics are given in Table 1. AML is a very heterogeneous disease and not all patient's blasts are sensitive to analogs [24]. Therefore, to compare activities of the analogs, out of nine patients we have chosen five, whose cells were sensitive to 1,25D-induced differentiation. As a measure of sensitivity we considered an increase of CD14-positive cells of at least 10 percentage points, after exposure to 1,25D. The results of differentiation experiments are given in Fig. 2C (1 nM analogs) and D (10 nM analogs). As presented, differentiation induced by 10 nM analogs is higher than that induced by 1 nM analogs, and at 10 nM concentration, PRI-1906 seems to be the most active.

3.3. Expression of VDR and C/EBPβ in cells exposed to 1,25D and analogs

The ligand-induced translocation of VDR into the nucleus is a critical step required for transcriptional activity of VDR [25]. We [26,27] and others [28,29], showed that nuclear accumulation of VDR is directly related to biological activity of 1,25D analogs.

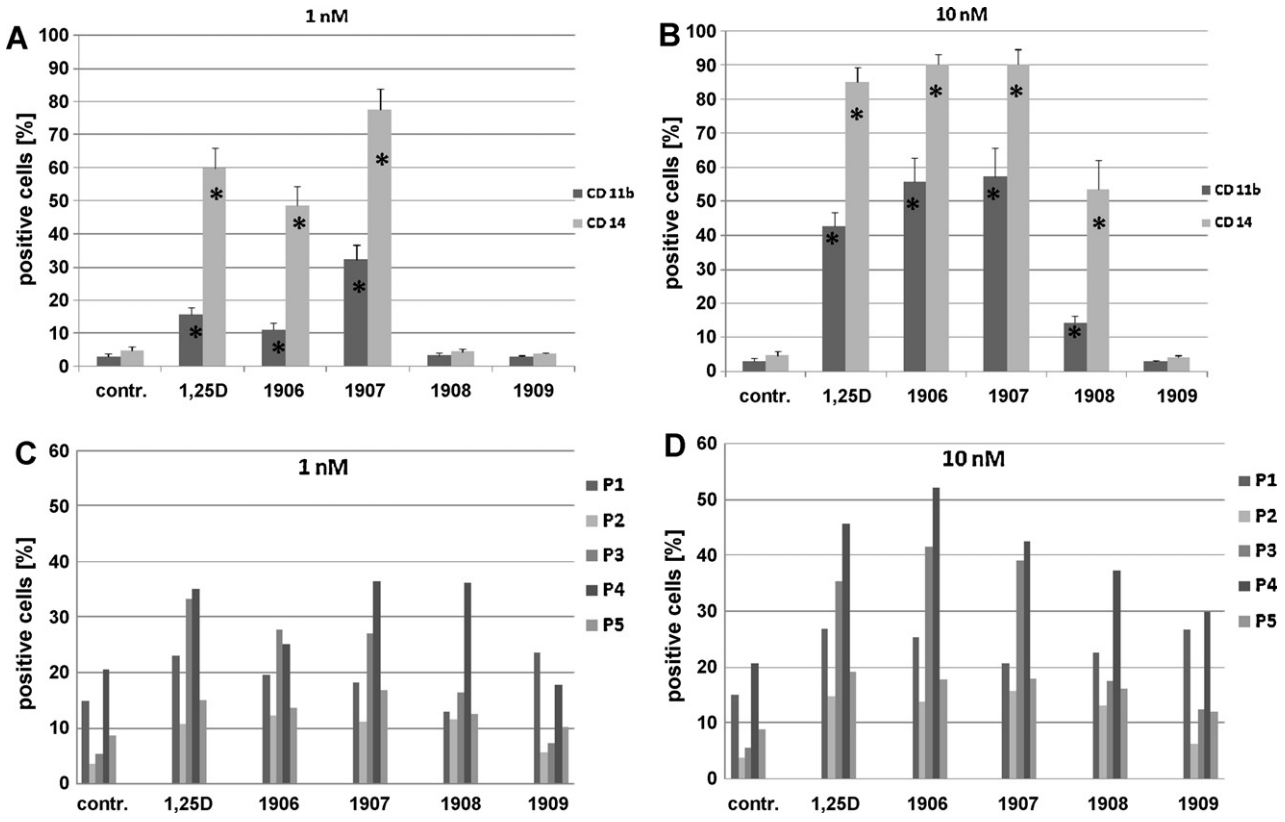


Fig. 2. Preliminary screening of pro-differentiating activities of analogs studied. HL60 cells (A and B) and blast cells from patients with AML (C and D) were exposed to 1,25D and analogs at concentrations of 1 nM (A and C) and 10 nM (B and D). The expression CD11b and CD14 was studied in HL60 cells, and expression of CD14 in blast cells. Percentages of positive cells are presented in *Y*-axis. The samples that differ significantly from the control are marked with asterisk ($p < 0.01$). P1; P2; P3; P4; P5 – number of probe of blast cells isolated from patients with AML.

Given that VDR nuclear accumulation and pro-differentiating activity were correlated for previously tested analogs [27], we now studied how vitamin D_2 analogs influence expression of VDR in HL60 cells. For this purpose we analyzed VDR expression in nuclear and cytosolic fractions of cells subjected to 24 h incubation with 10 nM compounds. The Hsp90 was detected as a protein that does not change during HL60 cells' differentiation. As it is visible from a representative experiment in Fig. 3A, all analogs with exception of PRI-1909 increased VDR levels as compared to vehicle, and induced accumulation of VDR in the nuclei of HL60 cells. VDR nuclear translocation induced by 1,25D, PRI-1906, and PRI-1907 was comparable one to another, and stronger than that observed for PRI-1908. The quantification is given below blots as a ratio of optical density of each VDR band versus respective Hsp90 band.

In addition, we have examined the ability of tested analogs to increase expression of C/EBPβ protein. This protein is one of the critical transcription factors involved in the monocytic differentiation of hematopoietic cells [30,31] and it is expressed in three different isoforms: C/EBPβ 1, 2 and 3 [32]. Our previous studies provided evidence that 1,25D up-regulates expression of this protein in HL60 cells [33], and down-regulation of C/EBPβ using antisense technology attenuated 1,25D-induced differenti-

Table 1
Characteristics of the patients.

Sample No	Age	Gender	FAB subtype/risk	Karyotype	Fusion gene/oncogene
P1	74	F	M5b/HR	46,XX[30]	nd
P2	56	M	M5a/HR	46,XY[20]	FLT3-ITD (−) NPM1 (+) MLL-PTD (−)
P3	70	F	M0/HR	47,XX,+21[4]/51,XX,+13,+14,+21,+21,+22 [12]/46,XX[14]	nd
P4	41	M	M2/HR	46,XY[20].ish11q23(5′MLLx,3′MLLx2) (5′MLLcon3′MLLx2)[8/10] Rearranged MLL – masked translocation t(6;11)	AML1-ETO (−) FLT3-ITD (−) NPM1 (−) MLL-PTD (−)
P5	20	M	M1/HR	46,XY[40]	AML1-ETO (−) FLT3-ITD (−) NPM1 (+) MLL-PTD (−) BCR-ABL (−)

nd, no data.

A

compound	Contr.		1,25D		1906		1907		1908		1909	
fraction	C	N	C	N	C	N	C	N	C	N	C	N
VDR→												
Hsp90→												
VDR/Hsp90	0.0	0.16	0.26	0.71	0.18	0.83	0.19	0.60	0.10	0.26	0.06	0.13

B

compound	Contr.		1,25D		1906		1907		1908		1909	
fraction	C	N	C	N	C	N	C	N	C	N	C	N
C/EBPβ1,2 →												
C/EBPβ3→												
Hsp90 →												
β1,2/Hsp90	0.53	0.71	1.15	1.85	1.0	2.53	0.89	2.33	0.74	0.76	0.15	0.15
β3/Hsp90	0.0	0.0	0.59	0.46	0.77	0.41	0.69	0.13	0.26	0.08	0.0	0.0

C

treatment	Contr.		1,25D	
fraction	C	N	C	N
p42 →				
Histone H1→				
Histone H3 →				
Hsp90 →				
Actin →				

Fig. 3. Intracellular content and localization of VDR and C/EBPβ protein in HL60 cells exposed to 1,25D or analogs. HL60 cells were exposed to 1,25D or analogs at 10 nM concentration for 24 h and then expression of VDR was determined in cytosolic (C) and nuclear (N) fractions (part A). In HL60 cells exposed to 1,25D or analogs at 10 nM concentration for 96 h, the expression of C/EBPβ isoforms was studied (part B). Controls for the absence of cross-contamination during the cell fractionation procedure (part C). The cells were fractionated into cytosolic (C) and nuclear (N) fractions, which were analyzed in Western blots, using anti-p42/Erk, anti-Histone H1, anti-Histone H3, anti-Hsp90 and anti-Actin antibodies.

ation [34]. It was previously shown that time-course of C/EBPβ expression in response to 1,25D treatment differs from that of VDR. It is undetectable at 24 h but it raises gradually to reach maximal levels at 72–96 h of the exposure [33]. Therefore, we determined C/EBPβ protein levels in nuclear and cytosolic fractions of HL60 cells exposed to 10 nM 1,25D and to analogs for 96 h. As expected, the amounts of all three isoforms of C/EBPβ increased in the cells exposed to 1,25D, PRI-1906, PRI-1907, and PRI-1908 but not to PRI-1909 (Fig. 3B). In addition to an increase in total levels of each C/EBPβ isoform, we also observed nuclear translocation of these proteins with all analogs except of PRI-1909. We also tested if our fractionation protocol resulted in separation of cytosolic (C) and nuclear (N) compartments (Fig. 3C). Histones H1 and H3 were found in the nuclear fraction, while p-42/Erk only in cytosol. Hsp90 and actin were present in both compartments.

Overall, our results indicate that pro-differentiating ability of tested analogs is correlated with their ability to induce expression and nuclear translocation of two transcription factors: VDR and C/EBPβ, which are involved in the process of monocytic differentiation. At the same time inability of PRI-1909 to induce nuclear translocation of VDR and C/EBPβ may partially explain its poor pro-differentiating effects (Fig. 2A and B).

3.4. Comparison of pro-differentiating activities of analogs using cell lines with various mutations characteristic for AML

For the next series of experiments we have used five cell lines derived from AMLs. All cell lines used (HL60, NB-4, U-937, MV4-11 and MOLM-13) have complex kariotypes and most of them carry mutations characteristic for various types of AML. NB-4 cells carry the t(15;17) PML-RARA fusion gene, which is characteristic for AML M3, U-937 carry translocation t(10;11) often seen in AML M5. Both MV4-11 and MOLM-13 have an internal tandem duplication in Flt3 gene [35], MV4-11 in one, and MOLM-13 in both allele. In addition, MV4-11 cells express fusion gene MLL-AF4, while MOLM-13 fusion gene MLL-AF9 [36]. Pro-differentiating activities of 1,25D, PRI-1906 and PRI-1907 were studied in flow cytometry, using these cells. Consistent with our expectations, we observed concentration-dependent increase in expression of both CD11b and CD14, but always lower in case of CD11b than of CD14. We noticed clear differences in the ability of analogs to induce CD11b and CD14 expression at concentrations about 1 nM. At this concentration, PRI-1906 and PRI-1907 induced expression of these differentiation markers stronger than 1,25D in all cell lines tested (Fig. 4). In HL60 cell line the pro-differentiating effect of PRI-1907 was the strongest of all compounds. At the higher concentrations PRI-1906 and PRI-1907 induced expression of differentiation markers to the similar extent. In addition, we observed a very little induction of CD11b expression in NB4, MV4-11, and MOLM13 cells following the treatment with analogs.

3.5. Expression of CYP24A1 mRNA in response to 1,25D and vitamin D_2 analogs

CYP24A1 is known to be the most potent target gene of 1,25D. Thus, we evaluated the expression of CYP24A1 mRNA in HL60 cells by semi-quantitative RT-PCR. We have determined kinetics of CYP24A1 mRNA expression in relation to 18S RNA level (which is stable). In this assay amplification of CYP24A1 and 18S RNA message is performed in the same test tube, which reduces errors. We found that in untreated cells, mRNA for CYP24A1 is at very low levels, and it slowly grows after exposure to 10 nM 1,25D or analogs reaching maximum levels at 96 h. As an example, the expression level of CYP24A1 mRNA related to the expression of 18S RNA induced by exposure of HL60 cells to 10 nM PRI-1907, is presented in Fig. 5A. To quantify mRNA levels, the next series of experiments was performed using SYBR Green Real Time PCR. In these experiments levels of CYP24A1 mRNA were measured in relation to the levels of GAPDH mRNA (Fig. 5B). We could statistically compare expression of CYP24A1 mRNA induced by 1,25D, PRI-1906 and PRI-1907. There appeared that PRI-1907 induced significantly lower levels of CYP24A1 mRNA than 1,25D and than PRI-1906 after 96 h from the beginning of exposure.

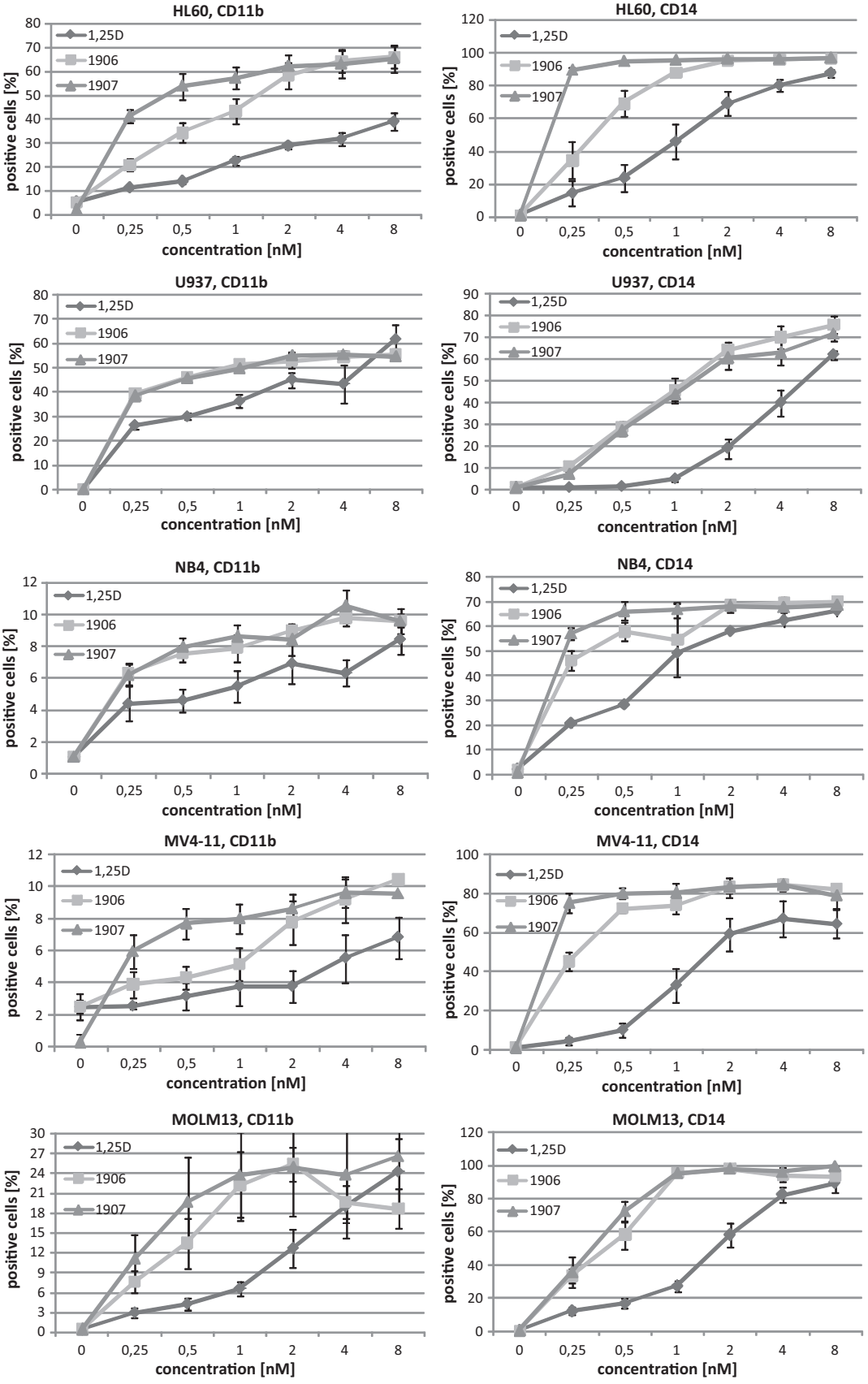


Fig. 4. Concentration-dependent differentiation effect of 1,25D, PRI-1906 and PRI-1907 towards AML derived cell lines. The cells were exposed to 1,25D, PRI-1906 and PRI-1907 for 96 h at concentrations of 0.25; 0.5; 1; 2; 4 and 8 nM, and then CD11b (left graphs) and CD14 (right graphs) cell surface markers were determined. Mean values (±SEM) of percentages of positive cells are presented in *Y*-axis. Names of cell lines are given in graph's titles.

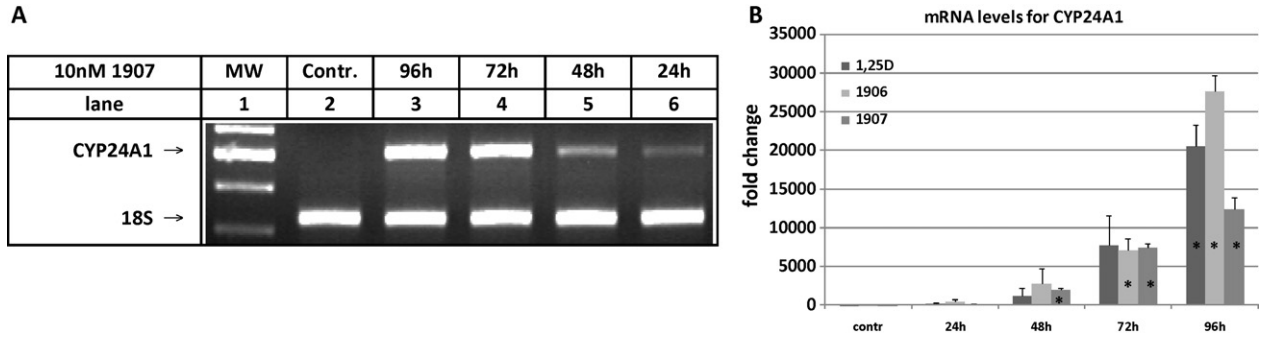


Fig. 5. Expression of CYP24A1 mRNA in response to 1,25D, PRI-1906 and PRI-1907. HL60 cells were exposed to 10 nM 1,25D, PRI-1906 and PRI-1907 for 24, 48, 72 and 96 h and then the expression of CYP24A1 mRNA was tested in a semi-quantitative RT-PCR (A) and in Real Time PCR (B). Expression of CYP24A1 related to the level of 18S RNA is presented in the representative gel on the left. The right graph shows mean values (±SEM) of fold changes in CYP24A1 mRNA levels relative to GAPDH mRNA levels. The samples that differ significantly from the control are marked with asterisk ($p < 0.01$).

3.6. Effects of 1,25D and vitamin D_2 analogs towards prostate cancer cells PC-3

Finally we wanted to verify, if similar structure–function relationship of the analogs tested as in leukemic cells exists in prostate cancer cells. For this purpose we used PC-3 cell line, and tested if analogs can inhibit proliferation of these cells, and if they induce expression of CYP24A1 mRNA. Anti-proliferative activity of analogs towards PC-3 cells was studied using Alamar blue assay. Fig. 6A shows that 1,25D, PRI-1906 and PRI-1907 induced approximately 30% inhibition of proliferation in PC-3 cells, while PRI-1908 and PRI-1909 did not have any effect (not shown). Since anti-proliferative activity of analogs tested was only moderate in PC-3 cells, structure–function relationship is less clear than in leukemic cells, however PRI-1908 and PRI-1909 again appeared to be less active. Then we studied kinetics of the expression of CYP24A1 mRNA in response to 1,25D and to two more active analogs in semi-quantitative PCR assay. Results presented in Fig. 6B show that PRI-1906 and PRI-1907 induce expression of CYP24A1 in PC-3 cells faster than 1,25D, and faster than in HL60 cells.

4. Discussion

Multiple studies have shown that 1,25D exerts anti-proliferative and pro-differentiating properties in many models of human cancer [37]. Many experiments using squamous cell carcinoma [38], prostate [39], breast [40] and lung cancers [41] showed *in vivo* anticancer effects of this compound. However, 1,25D is a potent regulator of calcium/phosphate metabolism, so when used in pharmacological concentrations, it can induce potentially fatal hypercalcemia. Therefore, to utilize therapeutic properties of 1,25D, it is necessary to obtain such analogs that would have increased benefits to risk ratio, as compared to 1,25D. As a result of this scientific quest, many different 1,25D analogs have been synthesized, which retain pro-differentiating activity of 1,25D, but have diminished calcemic effects [42].

The goal of this study was to determine the biological effects of analogs of vitamin D_2: PRI-1906, PRI-1907, PRI-1908, and PRI-1909 in human AML cell lines and in blasts from AML patients. PRI-1906 is a derivative of a vitamin D_2, which has been shown in previous studies to be less calcemic than 1,25D [43,18]. The initial *in vivo* studies have shown that PRI-1907 is more calcemic than PRI-1906 [18], whereas calcemic effects of PRI-1908 and PRI-1909 have not been studied yet. Our study indicates that among tested analogs the most interesting biological properties are possessed by PRI-1906 and PRI-1907. Experiments carried on AML derived cell lines indicate that PRI-1906 and PRI-1907 have comparable to each other, and stronger than 1,25D pro-differentiating activities. Similar conclusion could be drawn from the studies using blasts from AML patients, however, due to the limited sample number they cannot be supported by statistical analysis now. In addition, we have shown that these analogs efficiently induce elevated levels of VDR and C/EBPβ proteins which are implicated in pro-differentiating effects of 1,25D. Similar pattern of structure–function relationship was observed, when anti-proliferative activities of these compounds towards leukemic and breast cancer cell lines were studied

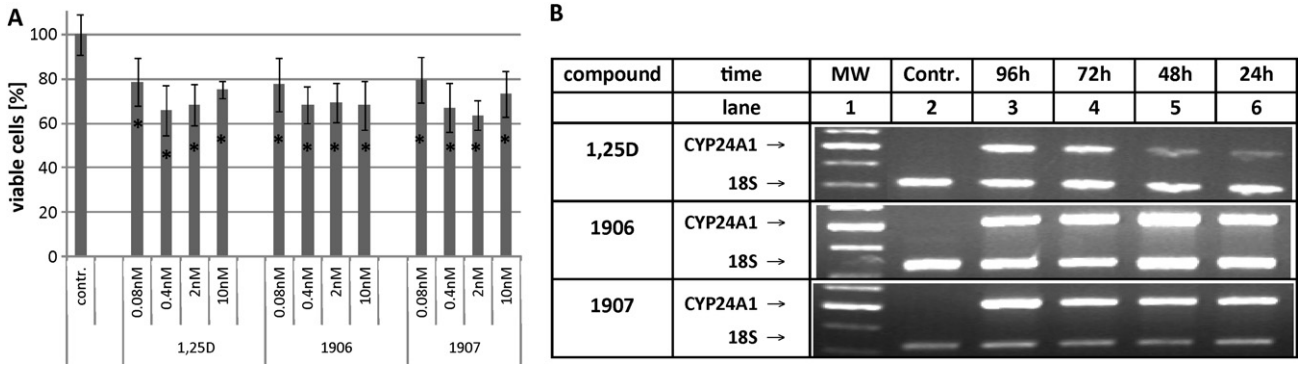


Fig. 6. Effects of vitamin D_2 analogs towards prostate cancer PC-3 cells. PC-3 cells were exposed to 1,25D and to analogs at concentrations of 0.08; 0.4; 2 and 10 nM for 96 h and the viability of cells was measured (A). Results for untreated (contr.) cells were considered as 100%. The viability of cells in treated samples is presented as a percentage of control. The samples that differ significantly from the control are marked with asterisk ($p < 0.01$). Expression level of CYP24A1 mRNA in response to 1,25D, PRI-1906 and PRI-1907 was tested in semi-quantitative RT-PCR assay in relation to 18S RNA(B). PC-3 cells were exposed to the compounds at 10 nM concentrations for 24, 48, 72 and 96 h. Representative gels for each compound are presented.

[16]. In all cell lines PRI-1906 and PRI-1907 were more active than PRI-1908 and PRI-1909, and in T47D human ductal breast carcinoma PRI-1907 was more active than both PRI-1906 and 1,25D [16].

Further, we showed that PRI-1906 and PRI-1907 have some biological effects in prostate cancer cells. In particular, we observed approximately 30% inhibition of proliferation in PC-3 cells in response to PRI-1906 and PRI-1907. It is of significance because this prostate adenocarcinoma cell line was described as weakly sensitive to the anti-proliferative action of 1,25D, due to reported high expression of 1,25D catabolising enzyme CYP24A1 [44]. In our experimental procedure basal expression of CYP24A1 has not been detected, but it grew rapidly after exposure of the cells to the vitamin D_2 analogs. A much faster up-regulation of CYP24A1 in PC-3 cells than in HL60 cells can be reasonable explanation of modest biological effects of PRI-1906 and PRI-1907 in this cell line.

Interestingly, we have found that PRI-1907 which had the strongest pro-differentiating effects in HL60 cells, was not the most effective inducer of CYP24A1 gene expression. At this point it is difficult to explain this discrepancy and further research is needed. It is possible that, besides VDR genomic pathways, PRI-1907 affects other intracellular signals, and their cumulative effects decide about the final outcome. Another likely explanation is that catabolism of PRI-1907 in HL60 cells is at lower level than that of other analogs, i.e. PRI-1906. The latter hypothesis can be tested in future with the use of CYP24A1 inhibitors.

Since analogs tested in this study differ only in the length of the side chains located at carbon C-25, we obtained important suggestions for the design of 1,25D analogs in future. We observed that gradual increase of side chain from methyl to ethyl was accompanied by initial increase in activity (from PRI-1906 to PRI-1907), followed by rapid loss of activity when n-propyl and n-butyl chains were introduced (PRI-1908 and PRI-1909). This structure–activity relation might suggest the appearance of steric or hydrophobic repulsions between the longer chains of the ligand and aminoacid residues at the VDR LBD. Based on our studies, one can conclude that the optimum length of the side-chain is between that of PRI-1906 and that of PRI-1907. Overall, our results suggest that side-chains of PRI-1906 and PRI-1907 represent structures which efficiently activate signal transduction pathways important for cell differentiation.

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References

[1] E. Mellanby, Experimental rickets. Medical Research (G.B.), Special Report Series SRS61 (4), 1921, pp. 1–78.
[2] D.M. McCarthy, J.F. San Miguel, H.C. Freake, P.M. Green, H. Zola, D. Catovsky, J.M. Goldman, 1,25-Dihydroxyvitamin D_3 inhibits proliferation of human promyelocytic leukaemia (HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells, Leuk. Res. 7 (1) (1983) 51–55.
[3] E. Abe, C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, T. Suda, Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D_3, Proc. Natl. Acad. Sci. U. S. A. 78 (8) (1981) 4990–4994.
[4] E. van Etten, C. Mathieu, Immunoregulation by 1,25-dihydroxyvitamin D_3: basic concepts, J. Steroid Biochem. Mol. Biol. 97 (1–2) (2005) 93–101.
[5] K.K. Deeb, D.L. Trump, C.S. Johnson, Vitamin D signalling pathways in cancer: potential for anticancer therapeutics, Nat. Rev. Cancer 7 (9) (2007) 684–700.
[6] K. Kragballe, Vitamin, D_3 and skin diseases, Arch. Dermatol. Res. 284 (1) (1992) S30–S36.
[7] A.S. Dusso, A.J. Brown, E. Slatopolsky, Vitamin D, Am. J. Physiol. Renal Physiol. 289 (1) (2005) F8–F28.
[8] M.T. Mizwicki, D. Menegaz, S. Yaghmaei, H.L. Henry, A.W. Norman, A molecular description of ligand binding to the two overlapping binding pockets of the nuclear vitamin D receptor (VDR): structure–function implications, J. Steroid Biochem. Mol. Biol. 121 (1–2) (2010) 98–105.
[9] E. Marcinkowska, A. Wiedlocha, C. Radzikowski, 1,25-Dihydroxyvitamin D_3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells, Biochem. Biophys. Res. Commun. 241 (2) (1997) 419–426.
[10] X. Wang, J. Rao, G.P. Studzinski, Inhibition of p38 MAP kinase activity up-regulates multiple MAP kinase pathways and potentiates 1,25-dihydroxyvitamin D_3-induced differentiation of human leukemia HL60 cells, Exp. Cell Res. 258 (2) (2000) 425–437.
[11] X. Wang, E. Gocek, C.G. Liu, G.P. Studzinski, MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D_3, Cell Cycle 8 (5) (2009) 736–741.
[12] E. Marcinkowska, A. Wiedlocha, C. Radzikowski, Evidence that phosphatidylinositol 3-kinase and p70S6K protein are involved in differentiation of HL-60 cells induced by calcitriol, Anticancer Res. 18 (5 A) (1998) 3507–3514.
[13] Y. Zhang, J. Zhang, G.P. Studzinski, AKT pathway is activated by 1, 25-dihydroxyvitamin D_3 and participates in its anti-apoptotic effect and cell cycle control in differentiating HL60 cells, Cell Cycle 5 (4) (2006) 447–451.
[14] C. Carlberg, F. Molnar, Detailed molecular understanding of agonistic and antagonistic vitamin D receptor ligands, Curr. Top. Med. Chem. 6 (12) (2006) 1243–1253.
[15] A. Opolski, J. Wietrzyk, A. Chrobak, E. Marcinkowska, E. Wojdat, A. Kutner, C. Radzikowski, Antiproliferative activity in vitro of side-chain analogues of calcitriol against various human normal and cancer cell lines, Anticancer Res. 19 (6b) (1999) 5217–5222.
[16] M. Chodynski, J. Wietrzyk, E. Marcinkowska, A. Opolski, W. Szelejewski, A. Kutner, Synthesis and antiproliferative activity of side-chain unsaturated and homologated analogs of 1,25-dihydroxyvitamin D_2 (24E)-(1S)-24-Dehydro-24a-homo-1,25-dihydroxyergocalciferol and congeners, Steroids 67 (9) (2002) 789–798.
[17] E. Marcinkowska, A. Kutner, Side-chain modified vitamin D analogs require activation of both PI3-K and erk1,2 signal transduction pathways to induce differentiation of human promyelocytic leukemia cells, Acta Biochim. Pol. 49 (2) (2002) 393–406.
[18] J. Wietrzyk, D. Nevozhay, M. Milczarek, B. Filip, A. Kutner, Toxicity and antitumor activity of the vitamin D analogs PRI-1906 and PRI-1907 in combined treatment with cyclophosphamide in a mouse mammary cancer model, Cancer Chemother. Pharmacol. 62 (5) (2008) 787–797.
[19] M. Cui, A. Klopot, Y. Jiang, J.C. Fleet, The effect of differentiation on 1 25 dihydroxyvitamin D-mediated gene expression in the enterocyte-like cell line, Caco-2, J. Cell. Physiol. 218 (1) (2009) 113–121.
[20] A. Kutner, M. Odrzywolska, M. Chodynski, J. Zorgdrager, J.P. van de Velde, Nowe analogi 1,25-dihydroksycholekalcyferolu i sposób ich otrzymywania. PL 190579 B1, 2005.
[21] K.L. Perlman, A. Kutner, J. Prahl, C. Smith, M. Inaba, H.K. Schnoes, H.F. DeLuca, 24-Homologated 1,25-dihydroxyvitamin D_3 compounds: separation of calcium and cell differentiation activities, Biochemistry 29 (1990) 190–196.
[22] R. Gallagher, S. Collins, J. Trujillo, K. McCredie, M. Ahearn, S. Tsai, R. Metzgar, G. Aulakh, R. Ting, F. Ruscetti, R. Gallo, Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia, Blood 54 (1979) 713–733.
[23] R. Morosetti, D.J. Park, A.M. Chumakov, I. Grillier, M. Shiohara, A.F. Gombart, T. Nakamaki, K. Weinberg, H.P. Koeffler, A novel, myeloid transcription factor, C/EBP epsilon, is upregulated during granulocytic, but not monocytic, differentiation, Blood 90 (7) (1997) 2591–2600.
[24] E. Gocek, M. Kielbinski, H. Baurska, O. Haus, A. Kutner, E. Marcinkowska, Different susceptibilities to 1,25-dihydroxyvitamin D_3-induced differentiation of AML cells carrying various mutations, Leuk. Res. 34 (5) (2010) 649–657.
[25] A. Racz, J. Barsony, Hormone-dependent translocation of vitamin D receptors is linked to transactivation, J. Biol. Chem. 274 (27) (1999) 19352–19360.
[26] E. Gocek, M. Kielbinski, E. Marcinkowska, Activation of intracellular signaling pathways is necessary for an increase in VDR expression and its nuclear translocation, FEBS Lett. 581 (9) (2007) 1751–1757.
[27] E. Gocek, M. Kielbinski, P. Wyłob, A. Kutner, E. Marcinkowska, Side-chain modified vitamin D analogs induce rapid accumulation of VDR in the cell nuclei proportionately to their differentiation-inducing potential, Steroids 73 (14) (2008) 1359–1366.
[28] A. Klopot, K.W. Hance, S. Peleg, J. Barsony, J.C. Fleet, Nucleo-cytoplasmic cycling of the vitamin D receptor in the enterocyte-like cell line, Caco-2, J. Cell. Biochem. 100 (3) (2007) 617–628.
[29] A. Ismail, C.V. Nguyen, A. Ahene, J.C. Fleet, M.R. Uskokovic, S. Peleg, Effect of cellular environment on the selective activation of the vitamin D receptor by 1alpha,25-dihydroxyvitamin D_3 and its analog 1alpha-fluoro-16-ene-20-epi-23-ene-26,27-bishomo-25-hydroxyvitamin D_3 (Ro-26-9228), Mol. Endocrinol. 18 (4) (2004) 874–887.
[30] T. Graf, Differentiation plasticity of hematopoietic cells, Blood 99 (9) (2002) 3089–3101.
[31] V. Poli, The role of C/EBP isoforms in the control of inflammatory and native immunity functions, J. Biol. Chem. 273 (45) (1998) 29279–29282.

[32] N.A. Timchenko, A.L. Welm, X. Lu, L.T. Timchenko, CUG repeat binding protein (CUGBP1) interacts with the 5′ region of C/EBPbeta mRNA and regulates translation of C/EBPbeta isoforms, Nucleic Acids Res. 27 (22) (1999) 4517–4525.
[33] E. Marcinkowska, E. Garay, E. Gocek, A. Chrobak, X. Wang, G.P. Studzinski, Regulation of C/EBPbeta isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D3, Exp. Cell Res. 312 (11) (2006) 2054–2065.
[34] Y. Ji, G.P. Studzinski, Retinoblastoma protein and CCAAT/enhancer-binding protein beta are required for 1,25-dihydroxyvitamin D_3-induced monocytic differentiation of HL60 cells, Cancer Res. 64 (1) (2004) 370–377.
[35] H. Quentmeier, J. Reinhardt, M. Zaborski, H.G. Drexler, FLT3 mutations in acute myeloid leukemia cell lines, Leukemia 17 (1) (2003) 120–124.
[36] http://www.dsmz.de/human_and_animal_cell_lines.
[37] E. Gocek, G.P. Studzinski, Vitamin D and differentiation in cancer, Crit. Rev. Clin. Lab. Sci. 46 (4) (2009) 190–209.
[38] M.C. McElwain, R.A. Modzelewski, W.D. Yu, D.M. Russell, C.S. Johnson, Vitamin D: an antiproliferative agent with potential for therapy of squamous cell carcinoma, Am. J. Otolaryngol. 18 (5) (1997) 293–298.
[39] R.H. Getzenberg, B.W. Light, P.E. Lapco, B.R. Konety, A.K. Nangia, J.S. Acierno, R. Dhir, Z. Shurin, R.S. Day, D.L. Trump, C.S. Johnson, Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system, Urology 50 (6) (1997) 999–1006.
[40] K.W. Colston, S.K. Chander, A.G. Mackay, R.C. Coombes, Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro, Biochem. Pharmacol. 44 (4) (1992) 693–702.
[41] K. Nakagawa, A. Kawaura, S. Kato, E. Takeda, T. Okano, 1 alpha,25-Dihydroxyvitamin D_3 is a preventive factor in the metastasis of lung cancer, Carcinogenesis 26 (2) (2005) 429–440.
[42] R. Bouillon, W.H. Okamura, A.W. Norman, Structure–function relationships in the vitamin D endocrine system, Endocr. Rev. 16 (2) (1995) 200–257.
[43] Y. Ji, A. Kutner, A. Verstuyf, L. Verlinden, G.P. Studzinski, Derivatives of vitamins D_2 and D_3 activate three MAPK pathways and upregulate pRb expression in differentiating HL60 cells, Cell Cycle 1 (6) (2002) 410–415.
[44] S.W. Yee, M.J. Campbell, C. Simons, Inhibition of vitamin D_3 metabolism enhances VDR signalling in androgen-independent prostate cancer cells, J. Steroid Biochem. Mol. Biol. 98 (4–5) (2006) 228–235.