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Studies on the mechanisms of superagonistic pro-differentiating activities of side-chain modified analogs of vitamin D_2

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Abstract. 1,25-Dihydroxyvitamin D_3 (1,25D) is implicated in many cellular functions including cell proliferation and differentiation, thus, exerting potential antitumor effects. A major limitation for therapeutic use of 1,25D are its potent calcemic and phosphatemic activities. Therefore, synthetic analogs of 1,25D for use in anticancer therapy should retain cell differentiating potential, with calcemic activity being reduced. Previously, we described pro-differentiating effects of vitamin D_2 analogs with extended and branched side-chains. Analogs with side-chains extended by a pair of one (PRI-1906) or two carbon units (PRI-1907) displayed elevated cell-differentiating activity towards some acute leukemia cell lines (AML) in comparison to 1,25D. In this study, the potential mechanism of this superagonistic activity of the analogs was addressed. At first, possible differences in the expression of CYP24A1, a major catabolizing enzyme for vitamin D compounds and resulting differences in the degradation of analogs were investigated. In addition, interactions of the analogs with vitamin D receptor (VDR) and resulting activation of CCAAT-enhancer-binding protein β (C/EBPβ) were studied. The results obtained show that superagonistic pro-differentiating activities of analogs PRI-1906 and PRI-1907 do not seem to be caused by their altered catabolism, but most probably by altered interactions with VDR and resulting downstream proteins.

Introduction

The most important role of 1,25-dihydroxyvitamin D_3 (1,25D) is to regulate calcium and phosphate levels by enhancing their intestinal absorption, renal reabsorption and by regulating bone mineralization. However, it is well documented that the actions of 1,25D extend beyond a role in mineral metabolism, as the compound is also important for cell proliferation (1), cell differentiation (2) and immunomodulation (3). Since differentiation of prostate cancer, breast cancer and myeloid leukemia cells may have beneficial therapeutic effects in pathological conditions, therapeutic applications for 1,25D have been postulated. Unfortunately, a major limitation for therapeutic use of 1,25D are its potent calcemic and phosphatemic activities. The doses of 1,25D, which are necessary to induce differentiation and inhibit cell proliferation *in vivo*, cause hypercalcemia and hyperphosphatemia that may be potentially life-threatening. Therefore there is a need of semi-selective 1,25D analogs that retain high differentiating and anti-proliferative activities with minimal or tolerable calcemic and phosphatemic activities (4).

1,25D is a steroid compound, which is either produced in skin from 7-dehydrocholesterol or delivered with food. There are two signal transduction pathways activated by 1,25D in target cells. The most important and the best documented is so called 'genomic pathway' that consists in activation of vitamin D receptor (VDR). VDR belongs to the superfamily of nuclear receptors for steroid and thyroid hormones. In order to be active VDR heterodimerizes with the retinoid X receptor (RXR). VDR upon ligation translocates to the cell nucleus and undergoes conformational changes, that allow binding to specific sequences called vitamin D response elements (VDRE) localized in promoter regions of target genes. Binding of 1,25D to VDR enhances heterodimerization with RXR and allows binding of the coactivators, known as vitamin D receptor-interacting protein complex (DRIP) (5). The complex includes histone acetylase which relaxes chromatin structure to make DNA accessible for RNA polymerase and to initiate transcription of target genes (6). The less precisely described is 'non-genomic pathway' which consists of intracellular signalling molecules, such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K) and others activated by a putative membrane VDR (mVDR) (7).

In our recent paper we described pro-differentiating effects of a series of vitamin D_2 analogs with extended and branched side-chains. Analogs with side-chains extended by one (PRI-1906) or two carbon units (PRI-1907) displayed elevated cell-differentiating activity in comparison to 1,25D (8). Moreover, previous studies in mice have shown that

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PRI-1906 is less calcemic, while PRI-1907 was more calcemic than 1,25D (9,10). Analogs PRI-1906 and PRI-1907 were more active than 1,25D in inducing cell differentiation of HL60, NB-4, MV4-11, U-937 and MOLM-13 acute myeloid leukemia (AML) cells. Moreover, towards some cell lines PRI-1907 was more active than PRI-1906, and both were more active than 1,25D. However, these three compounds had similar potency to inhibit proliferation of prostate cancer cells PC-3 (8). The ability of these analogs to induce expression of *CYP24A1* gene in HL60 cells was tested, and the increase in *CYP24A1* mRNA appeared to be lower in cells exposed to PRI-1907 than in cells exposed to either 1,25D or PRI-1906. The above mentioned results have shown that these two analogs are selective not only in calcemic and pro-differentiating activities, but also are differentialy active in different cell lines.

As it was discussed recently (11), the potential mechanisms through which selectivity of analogs could be achieved include interactions with serum transporting proteins, efficiency of cellular uptake, interactions with intracellular binding proteins, intracellular metabolism to inactive endproducts or to active intermediary metabolites, ligand-induced conformational changes in the VDR that affect binding to the DNA or to the complex of protein coregulators and eventually, activation of the non-genomic pathways through a putative mVDR. In case of our analogs the interactions with serum proteins could not have been of any importance, because experiments were done *in vitro*. All other mechanisms mentioned above could have an impact on selective biological actions of PRI-1906 and PRI-1907. Observed differences in regulation of CYP24A1 by our analogs in HL60 cells encouraged us to study if in other cell lines similar differences occur. We also wanted to verify if differences in *CYP24A1* mRNA levels in HL60 cells were followed by different CYP24A1 protein levels in the cell mitochondria. Eventually, in attempts to find the mechanism of selectivity of PRI-1906 and PRI-1907, we examined changes in expression and subcellular localization of VDR protein and downstream transcription factors in response to our analogs.

Materials and methods

Cell lines. HL60 cells were obtained from the European Collection of Cell Cultures. NB-4 cells were a kind gift from Professor G.P. Studzinski (University of Medicine and Dentistry of New Jersey). U-937, MV4-11 and MOLM-13 were purchased from German Ressource Center for Biological Material (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cells were propagated as suspension cultures in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). 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.

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 an 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-PE and isotypic control-PE were from ImmunoTools (Friesoythe, Germany). Chemiluminescence Blotting Substrate was from Roche Diagnostics (Mannheim, Germany). Mouse monoclonal anti-Hsp90, rabbit polyclonal anti-C/EBPβ and anti-VDR antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit IgG and anti-mouse IgG conjugated to peroxidase were from Jackson ImmunoResearch (West Grove, PA). Rabbit anti-actin antibodies were from Sigma.

Determination of cell differentiation. 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 1 μl of CD14-PE for 1 h on ice. Next, they were washed three times with ice-cold PBS and suspended in 0.5 ml PBS prior to analysis on FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA). The acquisition parameters were set for an isotype control. Data analysis was performed with use of WinMDI 2.8 software (freeware by Joseph Trotter). Differentiation assays were repeated from 3 to 5 times.

Preparation of cell lysates. In order to prepare cytosolic and nuclear lysates, the cells ($5x10^6$/sample) 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 18000 x g, at 4˚C. Supernatants were designated the cytoplasmic (C) fraction, and the nuclei remaining in pellets after one washing were sonicated for 10 sec in the same volume of lysis buffer as before (80 μl/$5x10^6$ cells). Following sonication nuclei were centrifuged again for 5 min, at 18000 x g, at 4˚C and the final supernatants were designated the nuclear (N) fraction. Samples were denatured by adding 20 μl of 5X sample buffer and boiling for 5 min.

In order to prepare cytosolic, membrane, nucleosolic and chromatin fractions $6x10^6$ cells/sample (equivalent of 20 μl packed cell volume) were washed with PBS and lysed using Pierce Subcellular Protein Fractionation kit according to the user's manual. Obtained lysates were denatured by adding 5X sample buffer (1/4 volume of the lysate) and boiled for 5 min.

To prepare mitochondrial extracts $2x10^7$ cells/sample were washed with PBS and then the mitochondria were isolated using Pierce Mitochondria Isolation kit for cultured cells according to the user's manual. Obtained mitochondrial pellets were lysed, denatured in 80 μl of 1X sample buffer and boiled for 5 min.

Western blot analysis. For western blotting 30 μl of cell lysates were separated on 12.5% SDS-PAGE gels and transferred to PVDF membranes. The membranes were then dried, and incubated sequentially with primary and a horseradish peroxidase-conjugated secondary antibody (1 h, room temperature).

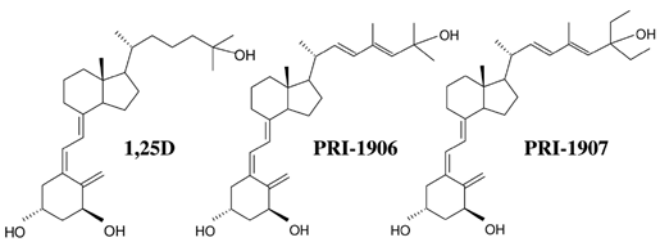

Figure 1. Structures of 1,25D and vitamin D_2 analogs. Chemical structures of compounds tested. The reference compound 1,25D is on the left. Side-chain modified analogs of vitamin D_2 are PRI-1906 (center) and PRI-1907 (right).

The protein bands were visualized with chemiluminescence. Then the membranes were stripped, dried again and probed with subsequent antibodies. Western blots were repeated from 2 to 5 times.

cDNA synthesis and real-time PCR. Total RNA was isolated using TriPure (Roche Diagnostics) as per 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). Real-time RT-PCR reaction was performed using SYBR-Green PCR Mix (A&A Biotechnology, Gdansk, Poland) and Applied Biosystems StepOne system. The sequences of CYP24A1 and GAPDH primers and reaction conditions were as described previously (8). Real-time PCR assays were repeated 3 times.

Results

Comparison of pro-diffrentiating activities of 1,25D and analogs. Previously, we showed that various AML cell lines when exposed to either 1,25D or vitamin D_2 analogs PRI-1906 and PRI-1907 (structures given in Fig. 1) acquire cell differentiation markers characteristic of monocytes. The most strongly upregulated is a key macrophage marker CD14, which functionally is a co-receptor for bacterial lipopolysaccharide (12). In order to compare potencies of these compounds in inducing cell differentiation we calculated from differentiation graphs the concentrations of the analogs that cause 50% ($C_{50\%}$) of treated cells to be CD14 positive. The values of $C_{50\%}$, given in Table I, show that in all cell lines studied PRI-1906 and PRI-1907 were more effective than 1,25D, what means that $C_{50\%}$ for PRI-1906 and PRI-1907 were from 4 to 14 times lower than $C_{50\%}$ for 1,25D. Moreover, in three cell lines (HL60, NB-4 and MV4-11) $C_{50\%}$ values for PRI-1907 were about 4 times lower than for PRI-1906. At concentrations around 10 nM, differentiating effects of the three compounds were similar, but when the analogs studied were applied at concentrations of 1 nM and lower, the differences in their potencies were evident.

Expression of CYP24A1 mRNA in response to 1,25D and analogs. *CYP24A1* gene encodes an enzyme, 24-hydroxylase of 1,25D, which is the key enzyme in degradation of 1,25D to calctirioic acid and it was documented that *CYP24A1* is the most strongly regulated out of all 1,25D-target genes (13). We have reported that in HL60 cells, the expression of *CYP24A1* was indeed very strong, but slowly upregulated in response to 1,25D (8). Surprisingly in HL60 the expression of *CYP24A1* cells was upregulated less by the analog PRI-1907 than by either 1,25D or PRI-1906. To determine whether similar pattern of regulation can be observed in other AML cells, for these studies two other AML cell lines were used. MV4-11 cell line, in which the analogs studied were similarly efficient in inducing CD14 expression as in HL60 cells, indicating that PRI-1907 was more active than PRI-1906 and both were more active than 1,25D and MOLM-13 cells in which PRI-1906 and PRI-1907 were similarly active, and both were more active than 1,25D. Since our previous experiments have shown that expression of mRNA for *CYP24A1* increases very slowly in response to 1,25D, for current experiments we selected 96 h of exposure to the compounds at 10 nM concentrations. Results which are presented in Fig. 2 show that unlike in HL60 cells, in MV4-11 and in MOLM-13 cells, analogs PRI-1906 and

Table I. Pro-diffrentiating activities of 1,25D and analogs towards human AML cell lines.

Compound	HL60	NB4	MV4-11	U-937	MOLM-13
1,25D	1.19	1.26	1.39	5.96	1.81
PRI-1906	0.39	0.43	0.40	1.09	0.39
PRI-1907	0.12	0.15	0.10	1.28	0.33

The cells were exposed to either 1,25D, PRI-1906 or PRI-1907 for 96 h and the expression of CD14 was detected using flow cytometry. Concentrations (nM) of the compounds that induce expression of CD14 cell surface marker on 50% of the cell population were calculated from differentiation graphs ($C_{50\%}$).

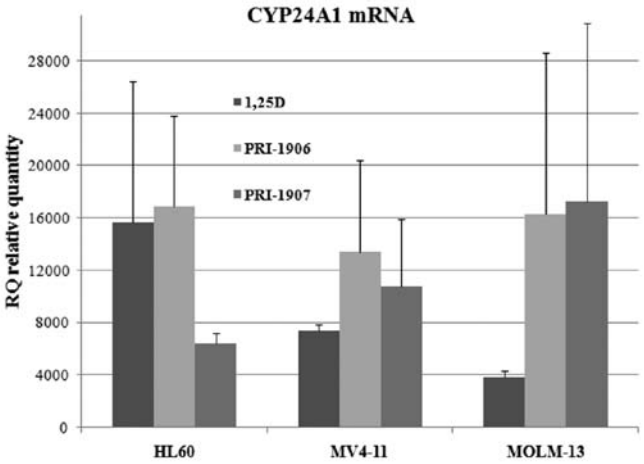

Figure 2. Expression of *CYP24A1* mRNA in HL60, MV4-11 and MOLM-13 cell lines. The cells were exposed to 10 nM 1,25D, PRI-1906 and PRI-1907 for 96 h and then the expression of *CYP24A1* mRNA was tested in real-time PCR. The graph shows mean values (±SEM) of fold changes in *CYP24A1* mRNA levels relative to GAPDH mRNA levels. The control samples were calculated as 1.

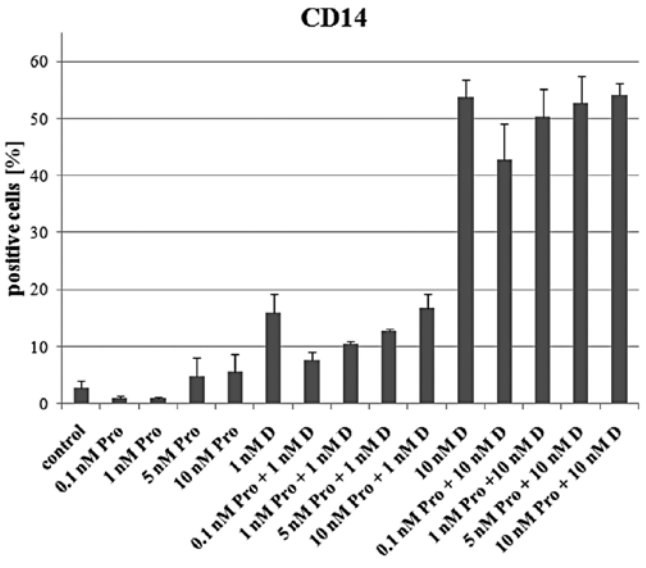

Figure 3. Expression of CD14 in HL60 cells exposed to 1,25D and CYP24A1 inhibitor. The cells were exposed to 1 or to 10 nM 1,25D (D) ± proadifen (Pro) at concentrations from 0.1 to 10 nM for 96 h and then the expression of CD14 was detected using flow cytometry. Mean values (±SEM) of percentages of positive cells are presented in Y-axis.

PRI-1907 upregulated expression of CYP24A1 stronger than 1,25D, however due to high standard deviations, differences were not significant.

Influence of CYP24A1 inhibitors to 1,25D-induced cell differentiation. Data obtained using prostate cancer models show that ketoconazole and other CYP24A1 inhibitors are able to enhance anti-proliferative action of 1,25D *in vitro* and *in vivo* (14-16). Since CYP24A1 is highly inducible by 1,25D in human AML cells, presumably resulting in an increased 1,25D degradation, an inhibition of the enzyme should cause its delayed catabolism and enhanced differentiation effect. Therefore in our next experiments HL60 cells were exposed simultaneously to 1,25D and various inhibitors of cytochrome P450 family of enzymes, which among others inhibit activity of CYP24A1. The inhibitors used were ketoconazole at concentrations ranging from 0.1 to 6 nM, proadifen at concentrations ranging from 0.1 to 10 nM and 1-aminobenzotriazole at concentrations ranging from 0.1 to 100 nM. The concentrations were verified as not toxic to the cells, before starting differentiation assays. The cells were exposed to either 1 or to 10 nM 1,25D ± the inhibitors given above. The cells were exposed for 96 h and then the expression of CD11b and CD14 was tested in flow cytometry. Surprisingly, neither inhibitor was able to induce any changes to 1,25D-induced expression of cell surface markers. As an example the graph showing expression of CD14 in cells exposed to 1,25D and proadifen is presented in Fig. 3. The values of CD14 expression in cells exposed to 1,25D and in cells exposed to proadifen and 1,25D are comparable.

Protein levels of CYP24A1 in HL60 cells exposed to 1,25D and analogs. In the next experiments we wanted to verify if differences seen in *CYP24A1* mRNA levels in HL60 cells are mirrored by CYP24A1 protein levels. Since CYP24A1 is a mitochondrial inner-membrane cytochrome P450 enzyme, in order to precisely identify its levels, mitochondria were isolated from HL60 cells exposed either to 1,25D or to the analogs. At first, the kinetics of CYP24A1 upregulation in cells exposed to 10 nM 1,25D was studied. Data from Fig. 4A show that CYP24A1 is expressed as a protein in HL60 cells at a very low, hardly detectable level, but after exposure to 1,25D it grows slowly, but significantly. Then the 96 h exposure-time was chosen to compare abilities of 1,25D and of the analogs to increase levels of CYP24A1 in mitochondria. As presented in Fig. 4B 1,25D, PRI-1906 and PRI-1907 at 10 nM concentrations induced comparable levels of CYP24A1 in HL60 cells. Then the analogs were applied at 1 and 0.1 nM concentrations, and CYP24A1 was detected in western blots. When the cells were exposed to 1 nM compounds, CYP24A1 was detectable, but levels induced by PRI-1906 and PRI-1907 were higher than levels induced by 1,25D (Fig. 4C). It appeared that levels of CYP24A1 in cells exposed to 0.1 nM compounds were similar to the control, and the western blot membranes had to be overexposed to see the bands (Fig. 4D).

Upregulation and subcellular localization of VDR and C/EBPβ in HL60 cells exposed to 1,25D and analogs. In light of the data presented above, the differences in expression of CYP24A1 do not seem responsible for different activities of 1,25D analogs. However, as mentioned before, differences in the analog activities were the most evident when compounds were applied at low concentrations (≤1 nM). Thus HL60 cells were exposed to 1,25D, PRI-1906 and PRI-1907 at 0.1 and 1 nM concentra-

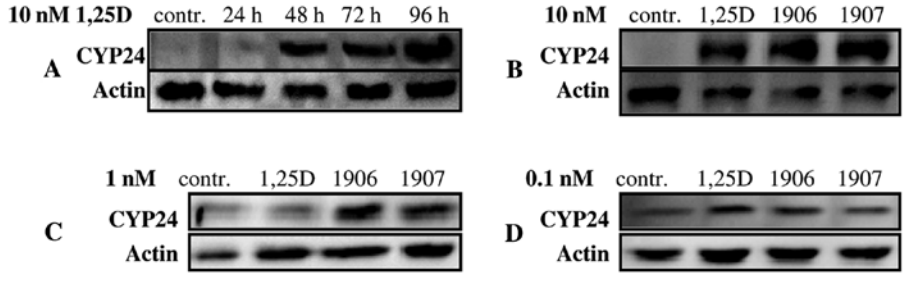

Figure 4. CYP24A1 protein in mitochondria of HL60 cells. HL60 cells were exposed to 1,25D for different times (A) and either to 1,25D or to analogs at 10 nM (B), 1 nM (C) and 0.1 nM (D) concentrations for 96 h. Then the expression of CYP24A1 in mitochondria was detected using western blot analysis. Mitochondria were isolated as described in Materials and methods. Mitochondrial proteins were analyzed in western blots, using anti-CYP24A1 and anti-actin antibodies.

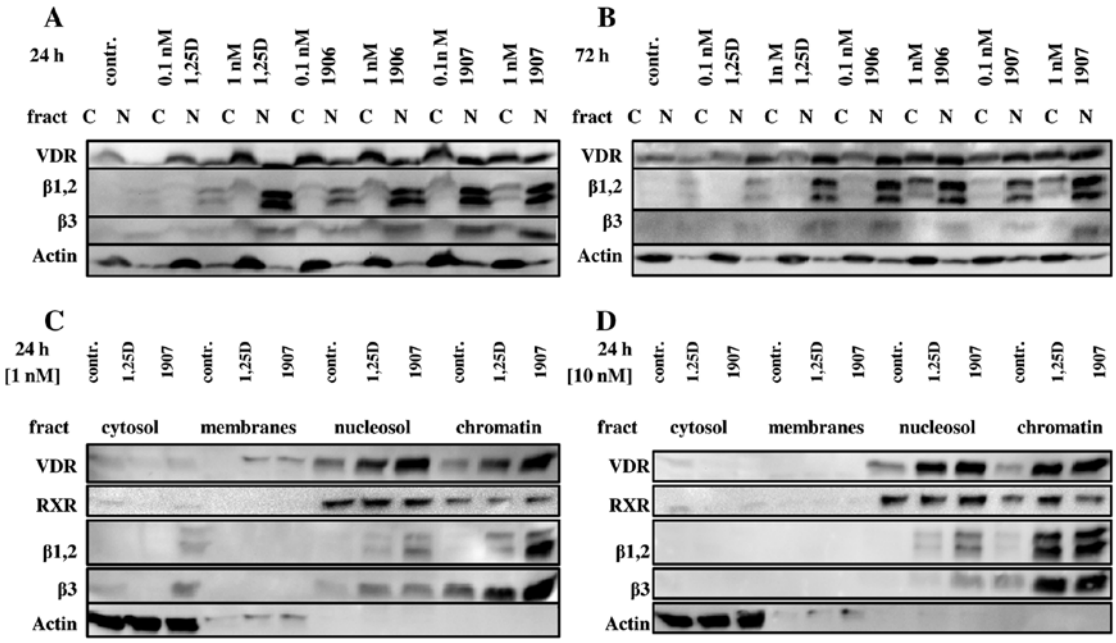

Figure 5. Subcellular distribution of transcription factors in HL60 cells. HL60 cells were exposed to 1,25D or analogs at 0.1, 1 and 10 nM concentrations for either 24 or 72 h and then expression of VDR, RXR and C/EBPβ isoforms was studied. The cells were fractionated into cytosolic (C) and nuclear (N) fractions (A and B) or into cytosol, membranes, nucleosol and chromatin fractions (C and D). Fractionated proteins were analysed in western blots using anti-VDR, anti-RXR anti-C/EBPβ and anti-actin antibodies.

tions, and the levels of VDR and CCAAT-enhancer-binding protein β (C/EBPβ) were tested in cell lysates. C/EBPβ was studied because this transcription factor is a master regulator of monocyte differentiation, and its upregulation and importance for differentiation of cells exposed to 1,25D were documented (17,18). C/EBPβ is translated from its mRNA in three isoforms of different lengths. The two larger isoforms, full length C/EBPβ-1 and slightly shorter version C/EBPβ-2 contain several transactivation domains, whereas the much shorter variant, C/EBPβ-3 lacks them (19). At first the expression of VDR and C/EBPβ was tested in HL60 cells fractionated into cytosol and nuclei. Previously we documented that this fractionation method efficiently separates cytosolic proteins from nuclear proteins (8,20). As presented in Fig. 5A and B, 0.1 nM 1,25D slightly increased nuclear content of VDR, and even less the levels of C/EBPβ isoforms. In contrast, 0.1 nM PRI-1907 strongly increased nuclear content of VDR and C/EBPβ, particularly after 24 h exposure. Also PRI-1906 applied at 0.1 nM concentration increased both VDR and C/EBPβ isoforms in nuclei of treated cells, predominantly after 72 h exposure. In the next series of experiments HL60 cells exposed either to 1,25D or to PRI-1907 were divided into more discrete cellular fractions using commercially available Pierce Subcellular Protein Fractionation kit, namely into cytosol, membranes, nucleosol and chromatin. When this fractionation protocol was applied, further differences were observed. First of all, almost equal distribution of VDR between nucleosol and chromatin was detected (Fig. 5C and D). When 1,25D and PRI-1907 were applied at 1 nM concentrations differences in VDR protein levels were clearly visible, but they were less evident when the compounds were used at 10 nM concentrations. Isoforms of C/EBPβ presented different pattern of distribution, as they were predominantly bound to chromatin. All isoforms of C/EBPβ were upregulated by the compounds studied, however 0.1 nM PRI-1907 was more efficient than 0.1 nM 1,25D in upregulation of chromatin-bound C/EBPβ isoforms.

Discussion

The major goal of many chemical laboratories is to develop vitamin D analogs that retain clinically useful activities of 1,25D with minimal calcemic and phosphatemic activities (4). There are some promising analogs that retain high pro-differentiating and anti-proliferative activities *in vitro* and express low calcemic activities *in vivo*. Even though many interesting analogs have been made, a clear pattern of structure-function relationship did not emerge (21). How complicated such structure-function relationships could be is well illustrated by a series of vitamin D_2 analogs with extended and branched side-chains (22). These analogs have an additional double bond in the side-chain, which is further extended by carbon units in an aliphatic and not alicyclic manner. The differentiating activity in AML cells increased from the analog with side-chain extended by a pair of methyl groups (PRI-1906), reaching maximum for the analog extended by a pair of ethyl groups at C-25 (PRI-1907). Further extensions of side-chain by either n-propyl (PRI-1908) or n-butyl (PRI-1909) groups caused sudden drop in pro-differentiating activities (8). Surprisingly, the minimum calcemic activity *in vivo* was observed for the analog PRI-1906, and was significantly lower than for 1,25D, as well as for PRI-1907 (10). Our earlier results suggested that the mechanism of superagonistic differentiating activity of PRI-1907 in HL60 cells could lie in its weak ability to upregulate expression of CYP24A1, a major catabolizing

enzyme for vitamin D. This hypothesis was addressed in the current study. However, the results presented here demonstrate that such explanation is very unlikely. First of all, in another cell line in which differentiating activity of PRI-1907 was stronger than the activities of 1,25D and PRI-1906, namely in MV4-11 cell line, levels of *CYP24A1* mRNA in response to PRI-1907 were higher than in response to 1,25D. Moreover, inhibition of catalytic activity of CYP24A1 protein using its inhibitors, have not increased 1,25D-induced monocytic differentiation, and finally the protein levels of CYP24A1 in HL60 cells exposed to 1,25D, PRI-1906 and PRI-1907 have not reflected mRNA levels detected in real-time PCR. We assume that it is important that mRNA and protein levels of CYP24A1 in AML cells increase very slowly after exposure of the cells to vitamin D compounds. The differentiation markers can be observed faster than CYP24A1 (23,24). Therefore we suppose that signals for differentiation are already 'on' when catabolism of vitamin D compounds starts. Consequently, we assume that altered catabolism of PRI-1907 is not a cause of its superagonistic differentiating actions in certain AML cell lines. There are some other possible mechanisms of superagonistic activities of analogs, for example higher efficiency of cellular uptake or altered interactions with intracellular binding proteins (11). Since the biggest differences between differentiating activities of 1,25D and PRI-1906 and PRI-1907 can be noticed when the compounds are applied at concentrations of 1 nM and less, we studied intracellular levels of VDR and C/EBPβ isoforms in HL60 cells exposed to 0.1 and 1 nM compounds. As was presented in our previous publications VDR is a rapid and sensitive indicator of intracellular presence of 1,25D and its analogs, as the protein is immediately stabilized and accumulated in cell nuclei after ligation (25,26). Therefore, the observation that nuclear levels of VDR are similar after exposure of the cells to either 0.1 nM PRI-1906 or 0.1 nM PRI-1907 as after 1 nM 1,25D may suggest that the two analogs are either more efficiently transported through cellular membrane, or have higher affinities to VDR. Nuclear accumulation of C/EBPβ transcription factor is more delayed response of the cells to 1,25D, as it can be observed not earlier than at 24 h, and reaches its maximum at 72 h from the exposure time (18). C/EBPβ is a regulator of monocytic differentiation, it directly regulates transcription of many monocyte-specific proteins, such as CD14, lactoferrin or lyzozyme (19). Moreover, recently an involvement of two longer isoforms C/EBPβ-1 and C/EBPβ-2 in differentiation-related inhibition of proliferation was reported (27). As presented herein, all three isoforms of C/EBPβ accumulate in the nuclei of cells exposed to 0.1 nM PRI-1906 or 0.1 nM PRI-1907, while for similar effect 1 nM 1,25D is needed. Almost entire nuclear content of C/EBPβ remains bound to chromatin, consequently suggesting transcriptional activity of these proteins. On the other hand the observation that at least half of the nuclear content of VDR remains in the soluble nucleosol, most probably in dimers with RXR, is surprising and suggests that the role of VDR in the nuclei of AML cells is not limited to transcription. In summary, we conclude that superagonistic differentiating activities of analogs PRI-1906 and PRI-1907 do not seem to be caused by their altered catabolism, but most probably by altered interactions with VDR and resulting downstream proteins.

Acknowledgements

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