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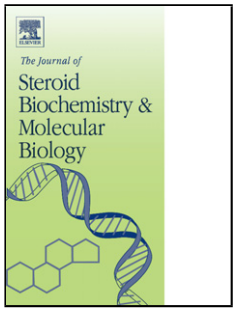

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BIOLOGICAL EVALUATION OF NEW VITAMIN D_2 ANALOGUES

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Highlights

> We examined activity profiles of double-point modified analogues of vitamin D_2.

> The analogues were less toxic *in vivo* than 1,25D.

> Pro-differentiating activities of analogues were stronger than that of 1,25D.

> The analogues upregulated expression of CYP24A1 and CD14 stronger than 1,25D.

> Neither calcemic, nor pro-differentiation effects were correlated to VDR binding.

Abstract

1,25-dihydroxyvitamin D_3 (1,25D), a steroid hormone which regulates calcium/phosphate homeostasis, has a broad spectrum of anti-cancer activities, including differentiation of acute myeloid leukemia (AML) cells. In order to avoid undesirable side effects such as hypercalcemia, low-calcemic analogues should be produced for therapeutic purposes. In this paper, we describe biological activities of double-point modified analogues of vitamin D_2 and we compare them to 1,25D and to paricalcitol, the drug used to treat secondary hyperparathyroidism. *In vivo*, our new analogues have lower calcemic effects, and lower toxicity in comparison to 1,25D. They have enhanced pro-differentiating and transcription-inducing activities in AML cells. Interestingly, differentiation effects do not correlate with the affinities of the analogues to the vitamin D receptor (VDR).

Keywords: vitamin D analogues; vitamin D receptor; leukemia; differentiation; calcemic effects; keratinocytes.

1. Introduction

1,25-dihydroxyvitamin D_3 (1,25D), a well known anti-rachitic agent [1], also has antitumor and pro-differentiating activities towards cancer cells [2, 3]. This is why many 1,25D analogues with improved anti-proliferative and pro-differentiating activities, as well as lower calcemic effects have been designed [4, 5]. Mechanistic studies of 1,25D analogues provide important information that allow us to determine which structural modifications of the 1,25D molecule are responsible for their changed biological properties. 1,25D exerts most of its biological functions *via* the vitamin D receptor (VDR) [6]. 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. CYP24A1, the gene most strongly regulated by VDR, encodes an enzyme responsible for degradation of 1,25D [7]. Another primary VDR-target gene is CD14, a monocytic cell differentiation marker, and a co-receptor for bacterial lipopolysaccharide, characteristic for monocytes and macrophages [8, 9].

It has been long believed in the field that only subtle changes to the structure of vitamin D might result in beneficial changes in the activity profile. Therefore, all the vitamin D drug substances were modified in a single point of the molecule. This is why in our search for vitamin D analogues of selective activity profile we modified up to now the molecule exclusively in the aliphatic side-chain. The most active analogues we obtained contained one-carbon unit extended (24a-homo) and rigidified (conjugated diene) side-chain (PRI-1906), additionally homologated at both terminal carbons (PRI-1907).

In our attempt to lower the toxicity of our PRI-1907 and to increase its biological activity we have now synthesised new generation of analogues of 1,25-dihydroxyvitamin D_2 modified in two distinct parts of molecule. In these structures we have combined our optimised side-chain of PRI-1906 and PRI-1907 [10-12] with the known 19-*nor* modification. This modification was previously introduced in the structure of a drug substance (paricalcitol, PRI-5100) and its 24-*epi* analogue (PRI-5101) [13], and we use them as a reference in our experiments. In this

paper we present our *in vivo* and *in vitro* evaluation of the resulting analogues PRI-5201 and PRI-5202 compared to the previously obtained ones and to 1,25D. The structures of the analogues are presented in Fig. 1.

2. Materials and Methods

2.1. Chemicals and antibodies:

1,25D and analogues were synthesised at the Pharmaceutical Research Institute (Warsaw, Poland). Antibodies for flow cytometry were from ImmunoTools (Friesoythe, Germany). Antibodies for western blots and chemiluminescence blotting substrate were from Santa Cruz (Santa Cruz, CA).

2.2. Cell lines, flow cytometry and western blots:

HL60 cells (Institute of Immunology and Experimental Therapy in Wroclaw, Poland) and HaCat cells (Dr. Miguel Quintanilla, Instituto de Investigaciones Biomedicas Alberto Sols, Madrid, Spain) were cultured using standard conditions. HL60 cells were incubated, labeled and analysed by flow cytometry as we have described before [10]. Western blotting was performed using nuclear fractions from cells as previously described [10].

2.3. cDNA synthesis and Real time PCR:

Total RNA was isolated and transcribed into cDNA. Then Real time PCR reaction was performed as previously described [10].

2.4. Human VDR binding assay:

Binding affinity to VDR was evaluated using a PolarScreen™ Vitamin D Receptor Competitor Assay Kit under manufacturer conditions (Life Technologies). The polarised fluorescence was measured using Envision (Perkin-Elmer). All compounds were evaluated within the range 10^{-11} to 10^{-5} M, IC_{50} values were calculated using the average of values obtained.

2.5. Calcemic activity in vivo:

All animals studies were approved by the University of Santiago de Compostela Ethics Committee for Animal Experiments. Male CD-1 mice (age matched, between 6 and 8 weeks) were obtained from Charles River Laboratories (L'Arbresle, France). The compounds were resuspended in sesame oil and administered intraperitoneally (0.3µg/kg) every other day for 21 days. Ethanol resuspended in sesame oil was used as the control for this experiment. Serum calcium levels were measured a day after the last dose using the QuantiChom Calcium Assay Kit (BioAssay Systems, Hayward, CA, USA) following manufacturer's guidelines. The weight of mice was measured every week.

2.6. Statistical analysis:

All experiments were repeated at least three times. Values are expressed as means ± SD. Dose response curves for cell differentiation and competitive VDR binding, calculation of EC_{50} and IC_{50} values, as well as analysis of statistical significance (ANOVA followed by t-tests) were performed using GraphPad Prism 6 software (San Diego, CA, USA).

3. Results

3.1. Calcemic activities in vivo:

First, we tested calcemic activities of our compounds in mice. The calcium serum levels induced by 1,25D and by all analogues, compared to the vehicle (ethanol) treated mice, are presented in Fig. 2A. Since toxic effects are not always directly connected to calcemia induced by 1,25D or by the analogues, we also examined the extent to which administration of the compounds affected the body weight of mice. The changes in body weight are related to overall toxicity (Fig. 2B). The results show that all of the tested analogues are less calcemic than 1,25D. The analogues PRI-1907, and to lower extent PRI-5202, exerted some general toxicity, similarly to 1,25D, as they affected weight gain of the mice. It is noteworthy, that mice receiving analogue PRI-5201, showed no significant change in body weight when compared to vehicle-treated mice.

3.2. Binding of analogues to VDR:

Since VDR is the primary target for 1,25D and analogues in cells, it is important to study the affinities of given analogues to this protein [14]. The experiments were performed using a fluorescence polarisation (FP)-based competition assay. The binding of analogues to VDR was tested over a wide range of concentrations and was compared to binding of 1,25D to the receptor. Dose-response curves were plotted (data not shown), and IC_{50} values were calculated from these dose-response curves (Table 1).

3.3. Differentiation of HL60 cells:

HL60 cells were used to determine how the subtle changes introduced to the structure of analogues influenced their pro-differentiating activities [15, 16]. The cells were exposed to compounds at a wide concentration range for 96 h and then the expression of monocyte/macrophage differentiation markers CD11b and CD14 was studied using flow cytometry. Since treated cells expressed CD14 a greater extent than CD11b, we therefore present the data obtained for this differentiation marker. Dose-response curves allowed us to calculate EC_{50} value for each analogue (Table 1). This data confirmed our earlier findings that analogue PRI-1907 is about one order of magnitude more active than 1,25D [10, 12], and it also shows that the new analogues, PRI-5201 and PRI-5202 are even more active in inducing AML cell differentiation than PRI-1907.

3.4. Transcription-inducing activities of 1,25D and analogues:

1,25D and its analogues are ligands of VDR, a receptor which is a ligand-activated transcription factor. The activity of analogues can be measured by the levels of transcription which they induce. In order to compare the transcriptional activities of the analogues we tested the expression of CYP24A1 and CD14 genes in HL60 cells exposed for 48 h (CD14) and 96 h (CYP24A1) to the analogues at 1 nM and 10 nM (not shown) concentrations. The results of CYP24A1 expression are presented in Fig. 3A, and CD14 expression in Fig. 3B. Again, the analogues PRI-1907, PRI-5201 and PRI-5202 are more active than 1,25D, especially at 1 nM concentrations.

3.5. Nuclear translocation and accumulation of VDR in response to analogues:

The ligand-induced translocation of VDR into the nucleus is a critical step required for transcriptional activity of VDR [17]. Given that VDR nuclear accumulation and pro-differentiating activity were correlated for previously tested analogues, we therefore studied how new analogues influence the levels of VDR protein in HL60 cells. We analysed VDR levels in nuclear fractions of cells exposed for different times to analogues at 1 nM concentration. Actin was used as a control, as it is a protein that does not change during HL60 cell differentiation. A significant increase in the level of VDR in the nucleus was seen as early as 3 h after exposing the cells to analogues, but at this time-point the VDR level does not correlate with differentiation-inducing activity of the given analogue. At later time-points, from 24 h (not shown) to 72 h post treatment, the amount of VDR in the nucleus correlates with the differentiation effect (Fig.4).

3.6. Activation of C/EBPβ by 1,25D and analogues:

In addition, we have examined the ability of tested analogues to increase expression of C/EBPβ protein. Our previous studies provided strong experimental evidence that 1,25D up-regulates expression of this protein in HL60 cells [18] and, it has been shown that down-regulation of C/EBPβ protein levels, using antisense technology, attenuates 1,25D-induced differentiation in this cell line [19, 20]. C/EBPβ expression in response to 1,25D treatment is undetectable at 24 h but it rises gradually to reach maximal levels at 3-4 days of treatment [18]. Therefore, we determined C/EBPβ protein levels in nuclear fractions of HL60 cells treated for 72 h with 1 nM 1,25D and analogues. The results are presented as western blots in Fig.5 which show that increased nuclear expression of C/EBPβ2 and C/EBPβ3 correlates well with the final cell differentiation effect.

3.7. Pro-differentiating activity of analogues towards human keratinocytes:

In order to determine whether the increased potency of analogues was limited to AML cells, we tested the differentiation effects of analogues towards human keratinocyte cell line HaCat [21]. After exposure to 1,25D and to other VDR ligands these cells change their growth properties and phenotype. This experiment showed in a qualitative manner the ability of analogues to induce differentiation of keratinocytes. The results presented in Figure 6 show that all the analogues induced phenotype changes to the HaCat cells which are characteristic for differentiated cells.

4. Discussion

1,25D is a very active compound with many biological activities, including induction of cell differentiation, immunomodulation and inhibition of cell proliferation [2, 3, 22]. 1,25D is also a potent regulator of calcium/phosphate metabolism, so when used at pharmacological concentrations, it can induce hypercalcemia [1]. To utilise the therapeutic properties of 1,25D, it is necessary to obtain analogues that would have increased benefits to risk ratio, as compared to 1,25D [5]. The analogues should be selective and should have increased pro-differentiating and anti-proliferative, and lowered calcemic activities. Regulation of the effective concentration and biological activity of 1,25D is maintained at multiple levels including transport of the analogue to the cell, intracellular localisation of the receptor, effective activation of transcription and degradation of 1,25D to inactive metabolites [23]. Therefore, it is necessary to complete a wide range of experiments to fully characterise new vitamin D analogues.

Our experiments revealed that the new, double-point modified analogues do not increase serum calcium levels as much as 1,25D. Their toxic effects, displayed as their ability to influence mice body weight, are lower than that of 1,25D, and also lower than toxicity induced by PRI-1907, the most active compound out of the single-point modified vitamin D_2 analogues. The pro-differentiating activities of PRI-5201 and PRI-5202 are more than an order of magnitude higher than that of 1,25D which suggests that they can be applied at lower concentrations to obtain anti-cancer or immunomodulatory effects. New, toxicological screening of these analogues, in a broader range of concentrations should reveal whether they are suitable for long-term therapy.

In our previous experiments, we have shown that the high differentiation-inducing potential of PRI-1907 correlated with its ability to increase the expression of the master regulator of monocyte differentiation, namely the C/EBPβ transcription factor and with upregulation of VDR target CD14 gene. The same correlation was observed for double-point modified

analogues (PRI-5201 and PRI-5202). The high pro-differentiating activities of PRI-5201 and PRI-5202, as compared to PRI-5100 and PRI-5101, cannot be explained by the binding affinities of these analogues for VDR. The affinity of PRI-5100 and PRI-5101 is more than four times higher than that of 1,25D. Interestingly, the affinity of PRI-5101, with the unnatural configuration at C-24 in the side-chain, is higher than that of PRI-5100 with the natural side-chain of 1,25-dihydroxyvitamin D_2. The affinity of the new analogue PRI-5201 is two times lower than that of PRI-5100 and the affinity of PRI-5202 is about seven times lower than that of PRI-5100 and PRI-5101. Therefore, we suggest that an interplay of different signal transduction pathways, genomic and non-genomic events, and the ability of given analogue to stabilise VDR in the cell nucleus of target cells (see Fig.4) are all important to eventual biological activity. The combination of selected structural fragments (side-chain of analogue PRI-1907 and 19-*nor* modification) has been shown to be very beneficial to the activity profile of vitamin D analogues. Until now analogues PRI-5201 and PRI-5202 represent the most active vitamin D compounds we designed, synthesised and biologically evaluated. Further modifications of the vitamin D structure leading to the same direction as to biological activity are under way in our laboratories.

5. Acknowledgements

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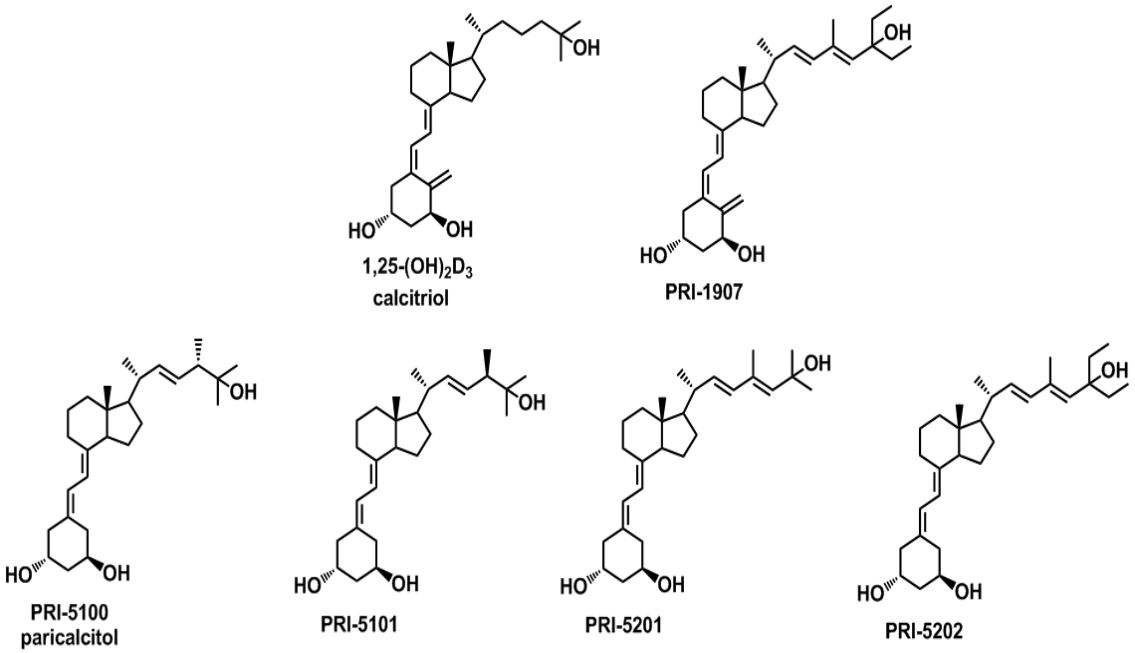

Fig. 1. Structures of 1,25D, PRI-1907, PRI-5100, PRI-5101, PRI-5201, PRI-5202.

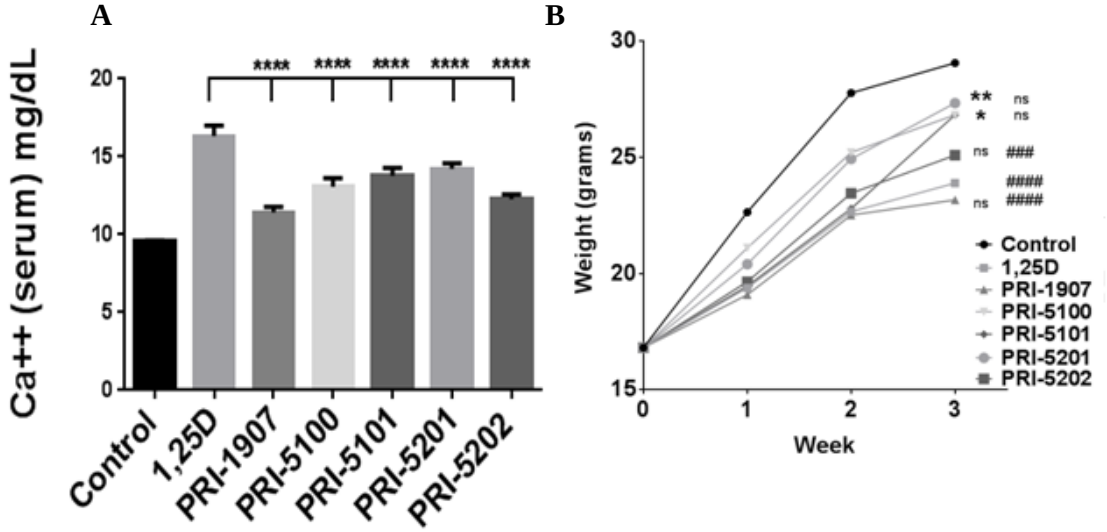

Fig. 2. *In vivo* effects of 1,25D and analogues.
A. Calcium levels in mice treated with the natural hormone 1,25D and compounds. Five mice per group were treated with 0.3 µg/kg of analogues, 1,25D or control every other day during 3 weeks, and calcium levels were measured on day 21**.** Error bars represent the standard deviation (SD). Analogues were compared to 1,25D, ****P ≤ 0.0001. B. Effect of analogues on body weight of mice. Mice were weighed every other day for 3 weeks. Analogues were compared to either the control (#) or to 1,25D (*) * P ≤0.05, **P ≤0.01, ### P ≤ 0.001, #### P≤0.0001, ns P > 0.05.

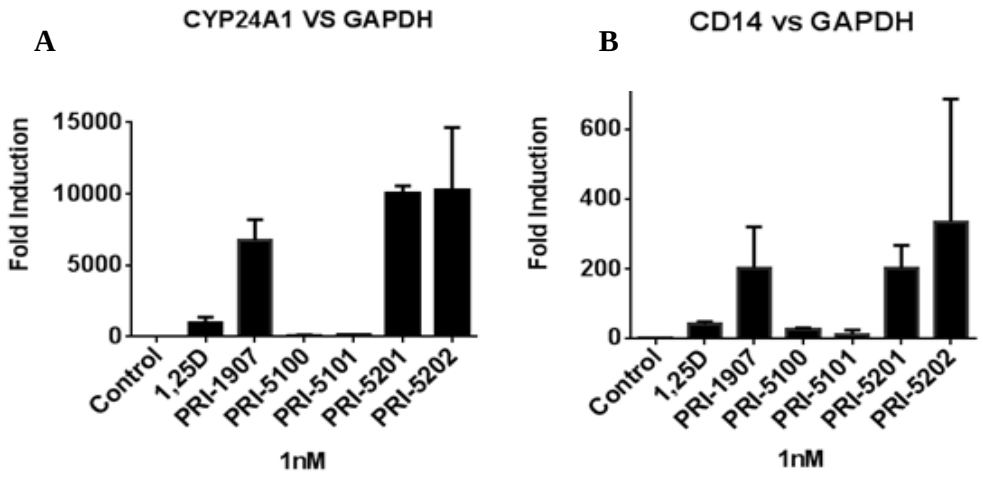

Fig. 3. Impact of 1,25D and analogues on mRNA levels.
A. HL60 cells were exposed to 1 nM 1,25D and analogues for 96 h and then the expression of CYP24A1 mRNA was tested in Real Time PCR. The graph shows mean values (±SEM) of fold changes in CYP24A1 mRNA levels relative to GAPDH. B. HL60 cells were exposed to 1 nM 1,25D and analogues for 48 h and then the expression of CD14 mRNA was tested in Real Time PCR. The graph shows mean values (±SEM) of fold changes in CD14 mRNA levels relative to GAPDH.

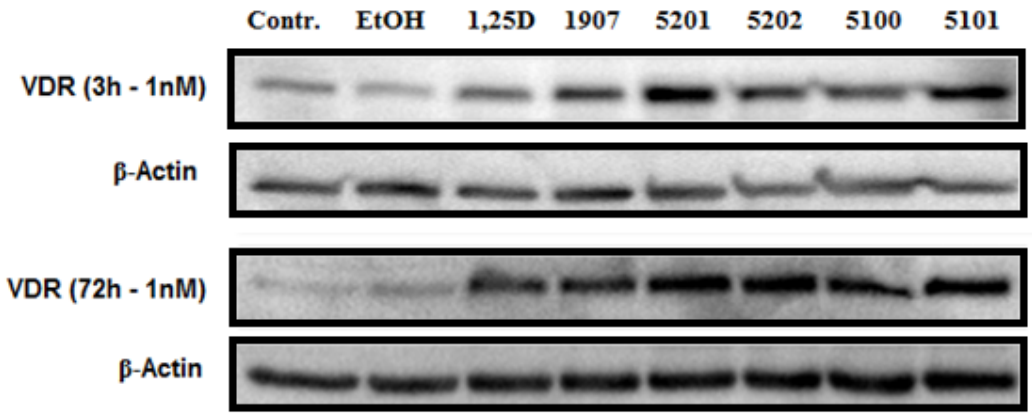

Fig. 4. Nuclear localisation of VDR protein in HL60 exposed to 1,25D or analogues.
HL60 cells were exposed to 1,25D or analogues at 1 nM concentration for 3 h and 72 h and then expression of VDR was determined in the nuclear fractions.

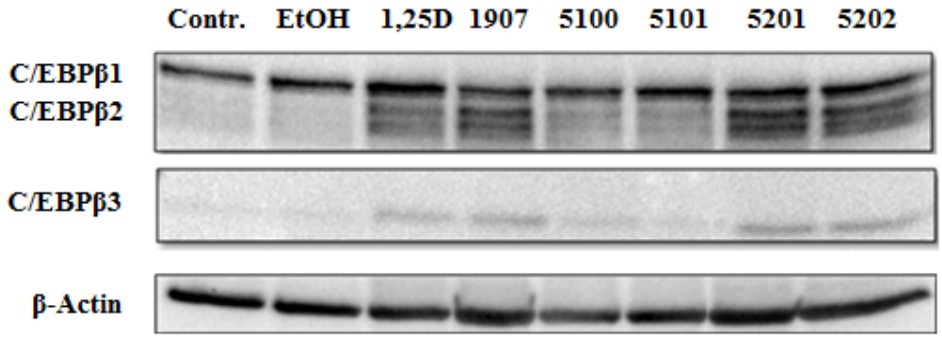

Fig. 5. C/EBPβ isoforms in HL60 cells treated with 1nM 1,25D and analogues.
HL60 cells were treated for 72 h with 1 nM 1,25D and analogues. The nuclear fraction was separated by electrophoresis and transferred onto PVDF membrane, and probed with antibodies against C/EBPβ, and β-actin as fractionation/loading controls. In addition to the three C/EBPβ isoforms, unidentified bands, possibly cleavage products of C/EBPβ, are present.

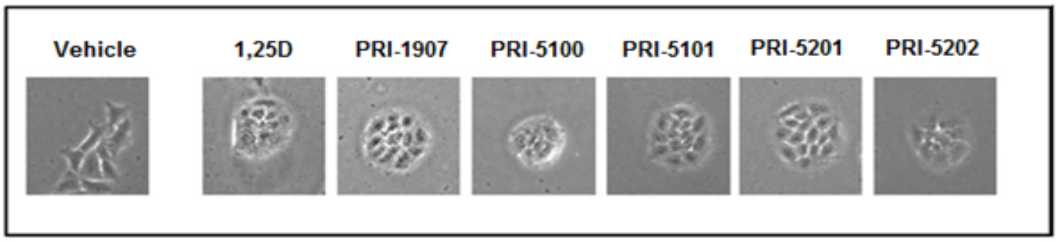

Fig. 6. Differentiation of HaCat cells in response to 1,25D or analogues.
Phase-contrast micrographs showing the induction by analogues of a differentiated adhesive epithelial phenotype in human HaCat cells. The cells were treated with analogues at 100 nM for 48 h.

Table. 1. VDR binding and differentiation-inducing activities of 1,25D and analogues PRI-1907, PRI-5100, PRI-5101, PRI-5201 and PRI-5202.

	VDR Binding		**Differentiation**	
	IC_{50} (M)	RBA[a]	EC_{50} (M)	EMR
1,25D	2.320 e^{-009}	100	5.347 e^{-010}	1
PRI-1907	6.172 e^{-009}	37	5.952 e^{-011}	0.11
PRI-5100	5.599 e^{-010}	414	1.128 e^{-009}	2.12
PRI-5101	4.921 e^{-010}	471	1.179e^{-009}	2.22
PRI-5201	1.193 e^{-009}	194	3.397 e^{-011}	0.063
PRI-5202	3.598 e^{-009}	64	1.788e^{-011}	0.033

The VDR binding affinity is expressed as IC_{50} and percentage activity. [a]The potency of 1,25D is normalised to 100. RBA: relative binding affinity. Cell differentiation activity is expressed as EC_{50} and EMR: effective molar ratio (EC_{50} analogue/EC_{50} 1,25D).