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Review

Novel analogs of 1,25-dihydroxyvitamin D_2 combined with a plant polyphenol as highly efficient inducers of differentiation in human acute myeloid leukemia cells

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ABSTRACT

1α,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is known to act as a powerful differentiation inducer in various types of cancer cells, including acute myeloid leukemia (AML) cells. However, supraphysiological concentrations of 1,25$(OH)_2D_3$ required to induce terminal maturation of AML cells can cause lethal hypercalcemia in vivo. Here we characterized the differentiation-inducing effects of novel double-point modified analogs of 1,25-dihydroxyvitamin D_2 [1,25$(OH)_2D_2$], PRI-5201 and PRI-5202 [Pietraszek et al. (2013) Steroids, 78:1003–1014], on HL60, U937 and MOLM-13 human AML cells in comparison with their direct precursors (PRI-1906 and PRI-1907, respectively) and 1,25$(OH)_2D_3$. The results demonstrated the following order of potency for the tested compounds: PRI-5202 > PRI-1907 > PRI-5201 > PRI-1906 ≥ 1,25$(OH)_2D_3$, as determined by measuring the expression of cell surface markers of myeloid differentiation. Particularly, the sensitivity of different AML cell lines to PRI-5201 and PRI-5202 was 3-15-fold and 13-50 fold higher, respectively, compared to that of 1,25$(OH)_2D_3$. Importantly, all the analogs tested at 0.25–1 nM concentrations retained the ability of 1,25$(OH)_2D_3$ to cooperate with the rosemary polyphenol carnosic acid, which strongly potentiated their prodifferentiation activity in a cell type-dependent manner. These synergistic effects were associated with increased induction of the vitamin D receptor (VDR) protein expression. However, surprisingly, carnosic acid was able to significantly enhance only 1,25$(OH)_2D_3$-induced transactivation of the direct repeat 3 (DR3)-type vitamin D response element (VDRE), whereas no such cooperation was seen with 1,25$(OH)_2D_2$ analogs. Furthermore, dose-response analysis revealed that 1,25$(OH)_2D_3$ was more efficacious than the analogs in inducing VDRE activation. This suggests that the superior prodifferentiation activity of the analogs, as compared to 1,25$(OH)_2D_3$, may be due to their potential for enhanced activation of the differentiation-related VDRE(s) that differ from the DR3-type element tested in this study. Collectively, the results demonstrate that the new double-point modified 1,25$(OH)_2D_2$ analogs are much stronger inducers of myeloid differentiation than 1,25$(OH)_2D_3$ and that their efficacy can be further enhanced by combination with plant polyphenols. These combinations warrant their further mechanistic and translational exploration in AML and other types of cancer.

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1. Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by a block of terminal differentiation of hematopoietic progenitors at early stages of myelopoiesis. This results in the accumulation of highly proliferative leukemic blasts in the bone marrow which disturbs normal hematopoiesis. Among the patients with newly diagnosed AML, 20–40% individuals do not fully respond to standard therapy with the cytotoxic drugs cytarabine and daunorubicin while 50–70% patients who achieve complete remission are expected to relapse within 3 years, and only about 10% of these patients will survive for 5 years [1]. Furthermore, elderly patients with AML are often ineligible for this treatment due to its toxicity and comorbidities, and outcomes for these patients are particularly poor [2]. Despite a number of experimental drugs developed for the therapy of AML most have failed in clinical trials. Except for gemtuzumab ozogamicin that has been recently withdrawn from the market, no new agent has yet been approved for AML in the last 40 years [3]. Hence the appeal of new sources for novel antileukemic drugs that may effectively and specifically target AML cells.

Differentiation therapy is an alternative AML treatment, based on the induction of leukemic blasts to mature beyond the differentiation block. The differentiation inducer all-*trans* retinoic acid has proven extremely valuable in the treatment of one subtype of AML, acute promyelocytic leukemia (APL) [4]. However, APL accounts for only ~10% of AML, and no differentiation therapy is currently available for other subtypes of AML. Vitamin D derivatives (VDDs), such as 1,25(OH)$_2$D$_3$, the hormonal form of vitamin D, and its synthetic low-calcemic analogs are known to regulate multiple cell events including cell proliferation, survival, differentiation, and immune responses [5,6]. The demonstration of marked antiproliferative and prodifferentiation effects of VDDs on AML cell lines and patient-derived leukemic blasts has suggested a potential therapeutic significance of these agents [6,7]. However, hypercalcemia induced by supra-physiological concentrations of VDDs still remains the major limiting factor for their clinical application [8].

A growing body of research indicates that combination strategy for VDD-based cancer therapy may prove more effective than monotherapy with these agents [6,9]. Preclinical studies in AML cells have shown that VDDs can potentiate growth arrest and cytotoxicity induced by chemotherapeutic agents [10,11]. On the other hand, various compounds, such as differentiation inducers (e.g., ATRA [9,12]), epigenetically active drugs (e.g., 5-aza-2′-deoxycytidine [13]), and anti-inflammatory agents [14,15] were found to enhance VDD-induced cell differentiation. Furthermore, we and others have shown that plant polyphenols, such as carnosic acid [16,17], curcumin [18,19] and silibinin [19,20], markedly potentiate the differentiation-inducing effects of near physiological concentrations of 1,25(OH)$_2$D$_3$ on AML cell lines and patient-derived leukemic blasts. We have also demonstrated that carnosic acid can synergistically enhance cell differentiation induced by low-calcemic 1,25(OH)$_2$D$_3$ analogs [21,22]. The latter findings may have clinical implications for the low-toxic combination differentiation therapy of AML.

We have previously reported the synthesis and evaluation of PRI-1906 and PRI-1907, the analogs of 1,25-dihydroxyvitamin D$_2$

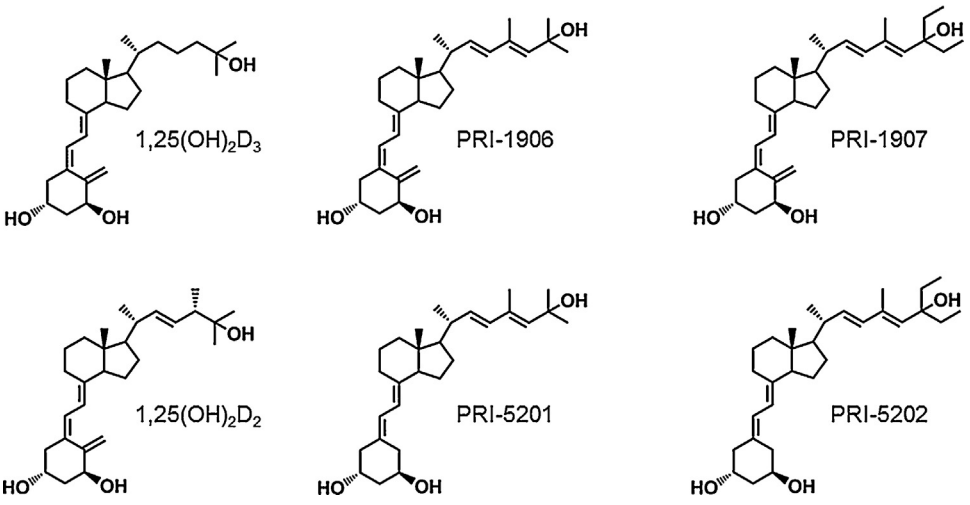

Fig. 1. Structures of the double point-modified analogs (PRI-5201 and PRI-5202) and their respective precursors (PRI-1906 and PRI-1907). Natural active forms of vitamin D$_3$ and D$_2$ are shown for comparison.

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($1,25(OH)_2D_2$) with increased ability to induce differentiation of AML cells compared to $1,25(OH)_2D_3$ [23,24]. Most recently we have synthesized the analogs, modified in the two distinct part of the vitamin D molecule (double point-modified analogs) [25]. Particularly, in the structures of PRI-5201 and PRI-5202 (Fig. 1) we combined the optimized length and unsaturation level of the side-chain of PRI-1906 and PRI-1907 and a very effective 19-*nor* modification [26]. In the present study, we characterized for the first time the anticancer effects of these novel compounds in vitro, employing cultured leukemia cell lines HL60, U937 and MOLM-13 that represent different subtypes of AML. The results demonstrated superior differentiation-inducing effects of PRI-5201 and PRI-5202 compared to the other compounds tested. In addition, the effects of all the tested $1,25(OH)_2D_2$ analogs at their low concentrations were potentiated by carnosic acid, which was selected for the combination studies due to its previously reported ability to markedly enhance VDD-induced differentiation of AML cells [16,17,21,22].

2. Materials and methods

2.1. Chemicals, antibodies and plasmids

Reference $1,25(OH)_2D_3$ and analogs PRI-1906, PRI-1907, PRI-5201 and PRI-5202 were synthesized at the Chemistry Department of the Pharmaceutical Research Institute (Warsaw, Poland). Carnosic acid (>98%) was obtained from Nanjing Chemlin Chemical Industry Co. (Nanjing, China). Antibodies against VDR (C-20), RXRα (D-20) and calregulin (H-170) were purchased from Santa Cruz Biotechnology (Dallas, TX). The VDRE × 6-Luc reporter construct containing a 6-fold direct repeat 3 (DR3) sequence [27] was kindly provided by Dr. L.P. Freedman (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). *Renilla* luciferase expression construct (pRL-null vector) was purchased from Promega (Madison, WI, USA) and served as an internal transfection standard.

2.2. Cell culture

HL60 myeloblastic leukemia cells (ATCC-CCL-240) were obtained from Dr. Rachel Levy (Ben-Gurion University of the Negev, Beer Sheva, Israel). U937 promonocytic leukemia cells (ATCC-CRL-1593.2) were purchased from American Type Culture Collection (Rockville, MD). MOLM-13 monocytic leukemia cells (ACC 554) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were grown in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 10 mM HEPES (pH 7.4) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. Determination of cell proliferation, viability and differentiation

Cells were seeded at 5×10^4 cells/ml and treated with test agents or vehicle (≤0.2% ethanol) for 96 h. Cell numbers and viability were determined on the basis of the trypan blue exclusion assay by enumerating in Vi-Cell XR cell viability analyzer (Beckman Coulter Inc., Fullerton, CA, USA). The number of viable (trypan blue-impermeable) cells was counted directly, and cell viability was calculated as the percentage of viable cells relative to the total (viable + dead) cell count. Aliquots of 5×10^5 cells were harvested, washed with PBS and incubated for 45 min at room temperature with 0.3 μL MO1-FITC and 0.3 μL MY4-RD1 (Beckman Coulter) to determine the expression of myeloid surface antigens CD11b and CD14, respectively, as described previously (e.g., [16]).

2.4. Preparation of whole cell lysates and western blotting

Cells were seeded at 1×10^5 cells/ml and incubated with test agents or vehicle (≤0.2% ethanol) for 48 h. Briefly, cells were lysed in 1% Triton X-100-containing buffer, subjected to SDS-PAGE and immunoblotting as described previously [17]. The VDR and RXRα antibodies were used at 1:200 and 1:1000 dilutions, respectively.

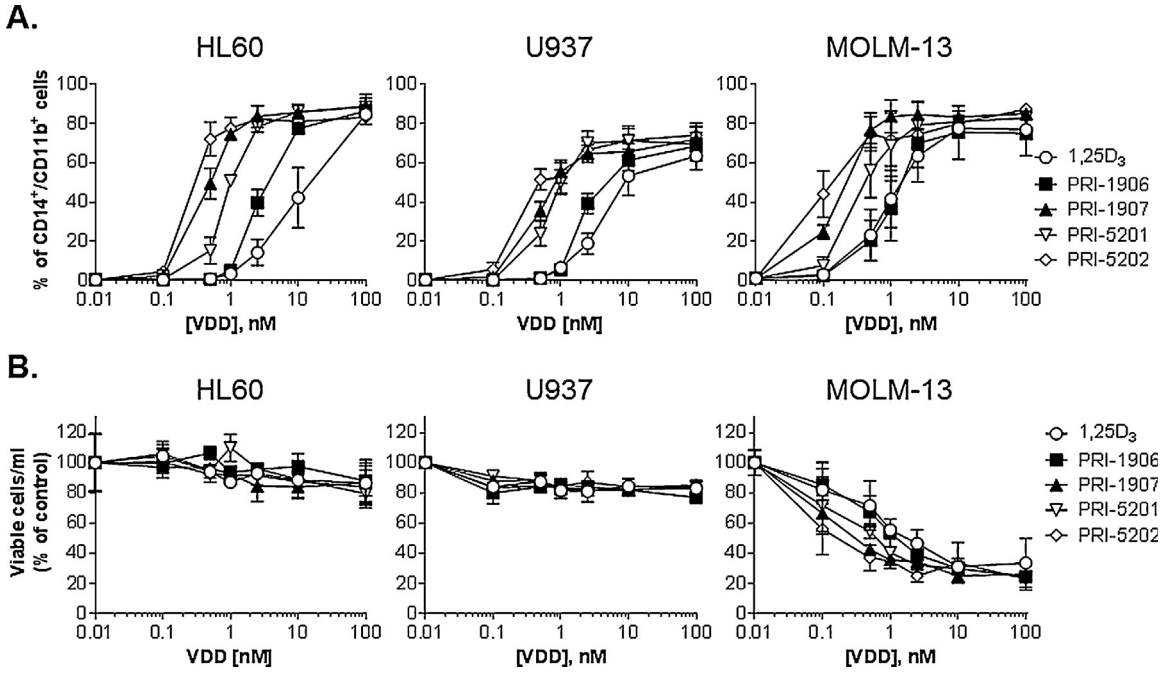

Fig. 2. Enhanced differentiation-inducing and cell type-specific antiproliferative effects of PRI-5201 and PRI-5202 on AML cells. Cells were incubated with the indicated agents or vehicle (≤0.2% ethanol) for 96 h. (A) The expression of CD14 and CD11b was determined by flow cytometry. (B) Cells were enumerated by the trypan blue exclusion assay. The data are the means ± SD ($n = 3$).

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Calregulin (1:500) was used as the internal loading control. The protein bands were visualized using the Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, MA). The optical density of each band was determined using the Image Quant LAS 4000 system (GE Healthcare Life Science).

2.5. Transient transfection and reporter gene assay

U937 cells were transiently co-transfected with the VDRE×6-luciferase reporter plasmid (0.8 μg) and *Renilla* luciferase vector (0.2 μg) using JetPEI Reagent (POLYplus-Transfection, Illkirch Cedex, France), as described previously [17]. Transfected cells were exposed to test agents for 24 h followed by measurement of luciferase activity using the Dual Luciferase Reporter Assay system (Promega). The data are presented as the normalized ratios of firefly luciferase to *Renilla* luciferase activity (relative luminescence units, RLU).

2.6. Statistical analysis

Experiments were repeated at least three times. EC_{50} values for the differentiation-inducing effects of VDDs were determined by nonlinear regression analysis of dose-response curves (variable slope; three or four parameters). Two compounds (A and B) were considered to show synergy in the particular experiment if the effect of their combination (AB) was larger than the sum of their individual effects (AB > A + B), the data being compared after subtraction of the respective control values from A, B, and AB [16]. The values are reported as the means ± SD. The significance of the differences between treatments was estimated by unpaired, two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with the GraphPad Prism 6.0 program (Graph-Pad Software, San Diego, CA).

3. Results and discussion

3.1. Comparative effects of a series of 1,25(OH)$_2$D$_2$ analogs on cell differentiation in different AML cell lines

We first determined the differentiation-inducing effects of novel double-point modified 1,25(OH)$_2$D$_2$ analogs, PRI-5201 and PRI-5202, on human AML cells in comparison with their direct precursors (PRI-1906 and PRI-1907, respectively) and 1,25(OH)$_2$D$_3$. These experiments were carried out using three AML cell lines representing different stages of myeloid differentiation: HL60 (myeloblastic leukemia, consistent with the AML-M2 subtype), U937 (myelomonocytic leukemia; AML-M4) and MOLM-13 (monocytic leukemia; AML-M5a). The extent of myeloid differentiation was assessed by bivariate analysis of surface expression of the specific monocytic marker CD14 and the general myeloid marker CD11b (Suppl. Fig. 1; Suppl. Table 1). Treatment with each VDD for 96 h resulted in a dose-dependent increase in cell surface levels of CD14 and CD11b (Suppl. Fig. 2) as well as in the percentage of cells expressing both myeloid antigens, i.e., $CD14^+/CD11b^+$ double-positive cells (Fig. 2A; Suppl. Fig. 1; Suppl. Table 1). All the cell lines tested demonstrated a similar order of VDD potencies (PRI-5202 > PRI-1907 > PRI-5201 > PRI-1906 ≥ 1,25(OH)$_2$D$_3$), as determined for the $CD14^+/CD11b^+$ cells (Table 1). Comparison of the EC_{50} values demonstrates that the potencies of PRI-5201 and PRI-5202 were higher relative to that of PRI-1906 and PRI-1907, respectively (Table 1). The difference between the potencies of PRI-5201 and PRI-1906 was more pronounced (~3–5-fold) compared to that between PRI-5202 and PRI-1907 (~1.5–2.0-fold). Importantly, in HL60 cells, PRI-5201 and PRI-5202 were about 15- and 50-fold more potent than 1,25(OH)$_2$D$_3$, respectively. In U937 cells, this difference was about 8- and 15-fold and in MOLM-13 cells, about 3- and 13-fold, respectively.

The prodifferentiation effects of PRI-1906 and PRI-1907 have been extensively studied in several AML cell lines and patient-derived leukemic blasts [23,28], and were shown to be more pronounced than those of 1,25(OH)$_2$D$_3$. The data presented here indicate that the ability of the new 1,25(OH)$_2$D$_2$ analogs to induce myeloid differentiation is even greater compared to the above compounds.

Table 1
Comparison of prodifferentiation potencies of different VDDs in AML cell lines.

Vitamin D derivatives	HL60	U937	MOLM-13
1,25(OH)$_2$D$_3$	12.86 ± 4.24	4.72 ± 1.01	0.93 ± 0.21
PRI-1906	2.67 ± 0.97	2.89 ± 0.77	0.97 ± 0.19
PRI-1907	0.44 ± 0.12	0.48 ± 0.15	0.16 ± 0.05
PRI-5201	0.89 ± 0.09*	0.59 ± 0.09*	0.34 ± 0.09*
PRI-5202	0.26 ± 0.03**	0.32 ± 0.02	0.07 ± 0.01**

EC_{50} values (nM) were calculated by non-linear regression analysis of the dose-response curves for the $CD14^+/CD11b^+$ double-positive cell population (Fig. 2A). The data are the means ± SD.
* $p < 0.05$ vs. PRI-1906.
** $p < 0.05$ vs. PRI-1907.

3.2. Effects of 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$ analogs on the growth of AML cells

It has been well documented that 1,25(OH)$_2$D$_3$-induced differentiation of AML cells is accompanied with G_1 cell cycle arrest, although the mechanisms underlying the two effects appear to be different (e.g., [29]). We, therefore, examined whether 1,25(OH)$_2$D$_2$ analogs can affect cell proliferation and/or viability. To this end, following incubations with the VDDs for 96 h viable and dead cells were enumerated using the trypan blue exclusion assay. We found that while all the VDDs strongly induced myeloid differentiation in all the cell lines tested, HL60 and U937 cells displayed only a modest reduction in cell numbers irrespective of the compound used (Fig. 2B), and without any change in cell viability (Suppl. Fig. 3). On the other hand, both 1,25(OH)$_2$D$_3$ and the analogs were capable of markedly inhibiting the growth of MOLM-13 cells (Fig. 2B), which was accompanied by a moderate decrease in cell viability (Suppl. Fig. 3). Interestingly, the order of antiproliferative activities of different VDDs in MOLM-13 cells was similar to that observed in the cell differentiation assays, i.e., PRI-5202 (IC_{50} = 0.04 nM) > PRI-1907 (IC_{50} = 0.11 nM) > PRI-5201 (IC_{50} = 0.14 nM) > PRI-1906 (IC_{50} = 0.59 nM) = 1,25(OH)$_2$D$_3$ (IC_{50} = 0.57 nM).

The reason for the marked difference in susceptibility of the three AML cell lines to the growth-inhibiting effects of VDDs is intriguing, particularly, since in contrast to HL60 and U937 cells, the MOLM-13 cell line expresses wild-type p53, a multi-functional transcription factor that regulates DNA repair, cell proliferation, cell cycle and apoptosis. Although we have previously shown that p53 status does not determine the differentiation-related G_1 cell cycle arrest induced by 1,25(OH)$_2$D$_3$ in AML cells [30], the role of this transcription factor in the regulation of myeloid leukemia cell growth by distinct VDDs remains to be investigated.

3.3. Enhancement of the antileukemic effects of 1,25(OH)$_2$D$_2$ analogs by carnosic acid

Potentiation of the antileukemic activity of 1,25(OH)$_2$D$_3$ and some of its analogs by plant polyphenols has been well documented (e.g., [16,18,20]); however, whether these botanicals can cooperate with the 1,25-dihydroxyvitamin D$_2$ analogs examined here has not yet been investigated. We, therefore, compared

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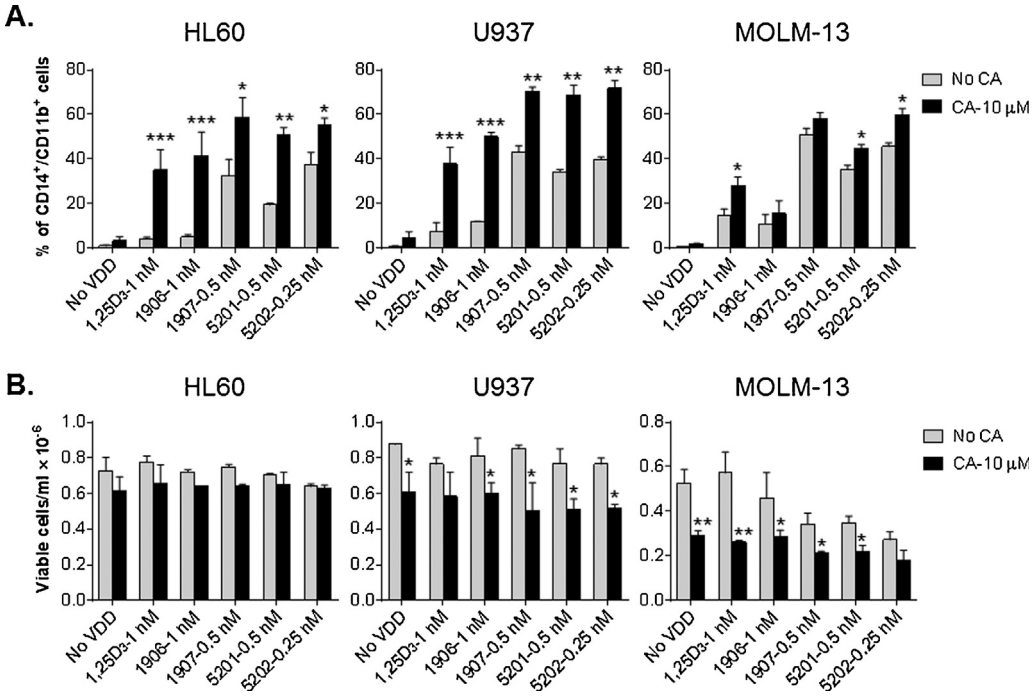

Fig. 3. Carnosic acid enhances differentiation-inducing effects of 1,25-dihydroxyvitamin D_2 analogs and inhibits proliferation in a cell type-specific manner. (A) The percentage of $CD14^+/CD11b^+$ cells was measured by flow cytometry. (B) The number of viable cells was determined by the trypan blue exclusion assay. The data are the means ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. the sum of the effects of single agents (A); and *, $p < 0.05$; **, $p < 0.01$ vs. VDD alone (B).

the extent of the differentiation and proliferation of HL60, U937 and MOLM-13 cells treated for 96 h with low concentrations of VDDs (0.25–1 nM), alone and in the presence of 10 μM carnosic acid. As shown in Fig. 3A, the untreated cell samples contained only a marginal percentage of the more mature $CD14^+/CD11b^+$ cells and carnosic acid alone had only a marginal effect on cell differentiation. However, combined treatments resulted in synergistically enhanced differentiation effects of $1,25(OH)_2D_3$ and analogs in all the cell lines tested, which occurred in both the VDD- and cell type-dependent manner. Similar results were obtained when the expression of CD14 and CD11b was analyzed separately (data not shown). Enumeration of cells in these experiments demonstrated that carnosic acid alone modestly reduced the numbers of HL60 cells but produced a more pronounced growth inhibition in the U937 and, particularly, MOLM-13 cell lines. Co-incubation with VDDs either did not further enhance this inhibition or produced slightly stronger inhibitory effects compared to the polyphenol alone (Fig. 3B).

These results indicate that the $1,25(OH)_2D_2$ analogs tested retain the ability of $1,25(OH)_2D_3$ to synergistically cooperate with

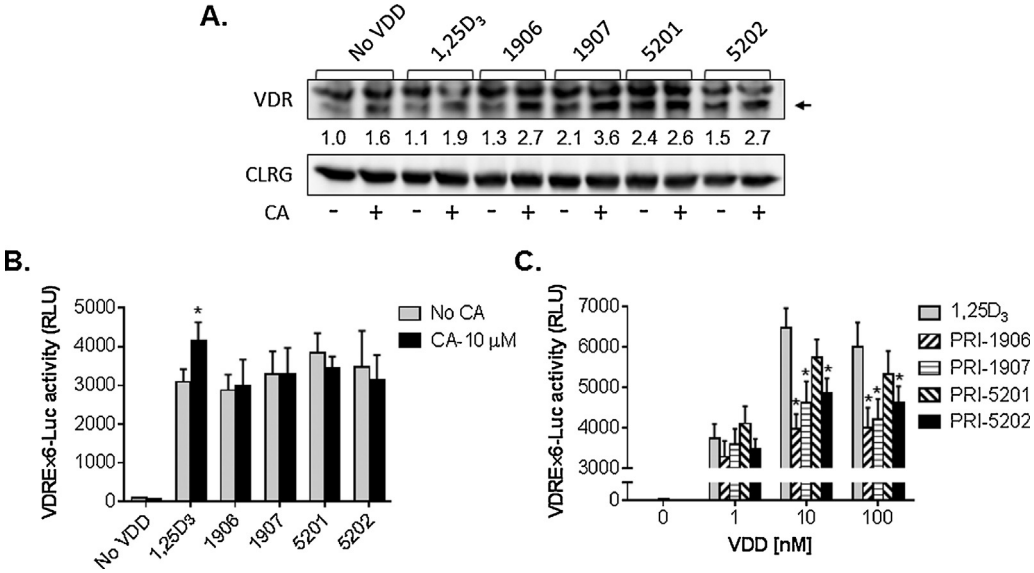

Fig. 4. Differential effects of $1,25(OH)_2D_3$ and 1,25-dihydroxyvitamin D_2 analogs, and their combinations with carnosic acid on VDR protein levels and VDRE transactivation in U937 cells. (A) Western blot assay following cell treatment with 10 μM carnosic acid and/or 1 nM VDDs, for 48 h. Relative VDR/calreguline (CLRG) ratios are indicated. A representative of 3 similar experiments is shown. (B and C) Cells transiently transfected with VDRE × 6-Luc and *Renilla* plasmids, and incubated with the indicated agents for 24 h. The relative VDRE × 6-Luc activity (means ± SD) was calculated from the data of 3 individual experiments performed in quadruplicate. *, $p < 0.05$, vs. $1,25(OH)_2D_3$ alone.

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carnosic acid and, possibly, with other plant polyphenols. This finding may have potential translational significance since we have previously reported that some low-calcemic analogs of 1,25$(OH)_2D_3$ and carnosic acid-rich rosemary extract synergistically retarded AML progression in mice without induction of hypercalcemia [21,22]. Importantly, several recent clinical studies in which VDDs were combined with differentiation-enhancing agents showed promising outcomes in patients with AML and myelodysplastic syndrome (MDS). For instance, in a retrospective case-control study, median survival of elderly patients treated with 25-hydroxyvitamin D_3 combined with the iron-chelating agent deferasirox was significantly increased in comparison with the patients receiving best supportive care [31]. In another study, a 4-year maintenance treatment with low-dose chemotherapy combined with 1,25$(OH)_2D_3$ and 13-*cis* retinoic acid in elderly patients with AML and MDS resulted in a lower relapse incidence and longer disease-free and overall survival compared to the patients who did not receive maintenance therapy [32].

3.4. Effects of VDDs and carnosic on the vitamin D receptor levels and activity in AML cells

Carnosic acid has been shown to modulate a wide array of cellular regulatory processes, including signaling kinase pathways, redox status and cell cycle control (reviewed in [33]). We have previously reported that the enhancing effect of carnosic acid on 1,25$(OH)_2D_3$-induced differentiation of AML cells is associated with the activation of the Raf-MEK-ERK1/2 [34] and JNK-AP-1-Egr-1 [19] pathways. Importantly, the carnosic acid/1,25$(OH)_2D_3$ combination also markedly upregulated the vitamin D receptor (VDR) levels and transcriptional activity through the activation of the transcription factors Nrf2 and AP-1 [17]. Here, using the U937 cell line as the model, we found that treatment with carnosic acid or a low concentration of each VDD (1 nM) alone resulted in an increase in VDR protein levels to the various extent (Fig. 4A). Under these conditions not only 1,25$(OH)_2D_3$ but also the analogs (except for PRI-5201) cooperated with the polyphenol to enhance VDR protein expression (Fig. 4A). The reporter gene assays performed in U937 cells transiently transfected with the vitamin D response element (VDRE)-luciferase construct demonstrated that, as expected [17], carnosic acid which alone was ineffective synergistically potentiated VDRE transactivation by 1 nM 1,25$(OH)_2D_3$ (Fig. 4B). Similar to 1,25$(OH)_2D_3$, 1,25$(OH)_2D_2$ analogs were capable of activating VDRE. However, surprisingly, carnosic did not significantly alter this activation (Fig. 4B). Further examination of this system revealed that while at 1 nM all the VDDs activated VDRE at a similar magnitude, at higher concentrations 1,25$(OH)_2D_3$ was clearly more efficacious (Fig. 4C).

The above data demonstrate the discrepancy between the inferior, with respect to 1,25$(OH)_2D_3$, ability of 1,25$(OH)_2D_2$ analogs, particularly, PRI-5201 and PRI-5202 to promote VDRE transactivation (Fig. 4C) and their superior potency in inducing the differentiation of AML cells (Fig. 2A). Furthermore, the failure of carnosic acid to potentiate VDRE activation by the analogs (Fig. 4B) is inconsistent with the facts that the polyphenol did enhance their ability to increase VDR levels (Fig. 4A) and to induce cell differentiation (Fig. 3A). These differences are intriguing and may suggest that the mechanisms by which 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$ analogs induce AML cells to differentiate are distinct at least in some aspects. One possibility, supported by findings reported by Carlberg and coauthors (e.g., [35,36]), is that the conformation of VDR modified by 1,25$(OH)_2D_2$ analogs may be different from that of the 1,25$(OH)_2D_3$-bound VDR. This may result in an enhanced selective transactivation by the analog-bound VDR/RXR complex of a subset of differentiation-related VDRE sequences that are different from the DR3-type VDRE present in the reporter construct used in this study. For instance, it was shown that a 100-fold higher antiproliferative potency of the 1,25$(OH)_2D_3$ analog EB1089, relative to 1,25$(OH)_2D_3$, in MCF-7 human breast cancer cells was due to an increased ability of EB1089 to transactivate the everted repeat (ER) ER9-type VDREs compared to DR3-type elements [35].

4. Conclusion

The principal and novel finding of this study is that the double point-modified 1,25$(OH)_2D_2$ analogs (PRI-5201 and PRI-5202) that have been recently synthesized by our group [26] exhibited a superior differentiation-inducing activity in a panel of AML cell lines compared to both 1,25$(OH)_2D_3$ and their direct precursors PRI-1906 and PRI-1907, respectively. This enhanced activity was achieved most likely by combining the synthetic modifications in the structure of the A-ring (19-*nor*) and in the side-chain (optimized length and unsaturation level) of PRI-1906 and PRI-1907. Moreover, we found that, similar to 1,25$(OH)_2D_3$, the analogs were capable to cooperate with carnosic acid to induce enhanced antileukemic effects concomitant with upregulation of VDR protein levels. The results of the VDRE transactivation assay suggest that the superior prodifferentiation effects of the analogs, as compared to 1,25$(OH)_2D_3$, may be due to enhanced analog-mediated activation of a specific subset of VDREs in the differentiation-related genes. These results provide the basis for further mechanistic and translational testing of the antileukemic activity of novel 1,25-dihydroxyvitamin D_2 analogs and their combinations with plant polyphenols.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.09.014.

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