ANTICANCER RESEARCH *36*: 6297-6302 (2016)
doi:10.21873/anticanres.11225

1α-Dihydroxyvitamin D_3 and Retinoic Acid Increase Nuclear Vitamin D Receptor Expression in Monocytic THP-1 Cells

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Abstract. *Background: 1α,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and retinoic acid, such as all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9cRA), are known to induce differentiation of myeloid leukemia cells. Combined treatment effectively enhances the differentiation effect, particularly in monocytic leukemia cells. The underlying mechanism of this combined effect remains unknown. Materials and Methods: THP-1 monocytic leukemia cells were treated with 1,25$(OH)_2D_3$ in combination with 9cRA, ATRA or selective synthetic ligand for retinoic acid receptor (RAR) or retinoid X receptor (RXR), and the nuclear expression and function of vitamin D receptor (VDR) were examined. Results: Combined treatment with 1,25$(OH)_2D_3$ and RAR ligand, not RXR ligand, effectively increased nuclear VDR expression and induced expression of the VDR target gene cathelicidin antimicrobial peptide (CAMP) in a gene-selective manner. Conclusion: Combination of 1,25$(OH)_2D_3$ plus RAR ligand is effective in induction of nuclear VDR expression and of target gene.*

The vitamin D receptor (VDR) belongs to the nuclear receptor superfamily and mediates biological actions of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], in calcium and bone metabolism, cellular growth and differentiation, immunity and cardiovascular function (1, 2). 1,25$(OH)_2D_3$ and its derivatives have been shown to inhibit the proliferation and induce the differentiation of various types of malignant cells, including myeloid leukemia cells (3). The administration of 1,25$(OH)_2D_3$ and its analogs was shown to have therapeutic effects in a mouse model of myeloid leukemia (4), and VDR ligands can induce the differentiation of various myeloid leukemia cells (5). Upon ligand binding, VDR undergoes a conformational change that results in interaction with the retinoid X receptor (RXR) and exchange of cofactor complexes (6). Ligand binding enhances nuclear localization of VDR to exert its transcriptional regulatory activity on specific target genes (7, 8). The VDR–RXR heterodimer binds preferentially to a consensus element that consists of a two-hexanucleotide (AGGTCA or a related sequence) motif separated by three nucleotides, called the direct repeat 3 (9). Such response elements are located in the VDR target gene promoters, including *CYP24A1* and *CAMP*, which encode vitamin D 24-hydroxylase and cathelicidin antimicrobial peptide, respectively. Ligand-bound VDR also mediates transrepression of genes, such as *CYP27B1* and *TNF*, which encode 25-hydroxyvitamin D 1α-hydroxylase and tumor necrosis factor α, respectively, through a poorly characterized mechanism. Although anti-leukemia effects of 1,25$(OH)_2D_3$ have been reported to be mediated by transactivation and transrepression, non-genomic actions are possible and the characterization of a detailed mechanism remains unclear (3, 5).

While 1,25$(OH)_2D_3$ induces monocytic differentiation of myeloid leukemia cells, all-*trans* retinoic acid (ATRA) induces their granulocytic differentiation (4, 10-12). ATRA also induces monocytic differentiation of monoblastic leukemia cells (13, 14). Combined treatment with 1,25$(OH)_2D_3$ and ATRA, or another natural retinoid 9-*cis* retinoic acid (9cRA), induces the differentiation of monoblastic leukemia cells to the monocyte/macrophage-lineage cells more effectively than does 1,25$(OH)_2D_3$ alone (15, 16). Interestingly, 9cRA plus 1,25$(OH)_2D_3$ induces M2 macrophage markers in differentiated monocytic leukemia cells (17). ATRA binds to the nuclear receptor retinoic acid receptor (RAR), while 9cRA acts as a ligand for both RAR and RXR (18). Although 1,25$(OH)_2D_3$ activates the VDR–RXR heterodimer effectively, this heterodimer does

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Key Words: VDR, vitamin D, retinoic acid, monocyte, antimicrobial peptide.

not allow RXR ligand activation (19). The combination of $1,25(OH)_2D_3$ with a selective RAR ligand induces differentiation of myeloid leukemia cells more potently than that with a selective RXR ligand (16). These findings indicate that retinoid signaling enhances differentiation induced by $1,25(OH)_2D_3$ through a mechanism different from activation of VDR–RXR heterodimer. In this study, we examined the effect of $1,25(OH)_2D_3$ in combination with retinoids on nuclear VDR expression.

Materials and Methods

Compounds. $1,25(OH)_2D_3$, ATRA and 9cRA were purchased from Wako Pure Chemical Industries (Osaka, Japan). A synthetic RAR agonist, Am80 (4-[(5,6,7,8-tetrahydro-5,5,8,8,-tetramethyl-2 naphthalenyl)]carbamoyl)benzoic acid), and a synthetic RXR agonist, HX630 (4-[2,3-(2,5-dimethyl-2,5-hexano)dibenzo[b,f][1,4]-thiazepin-11-y1] benzoic acid), were kindly provided by Dr. Koichi Shudo of Research Foundation ITSUU Laboratory (Tokyo, Japan).

Cell culture. THP-1 human myeloid leukemia cells (RIKEN Cell Bank, Tsukuba, Japan) were culture in RPMI-1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. Suspensions of cells (10^5 cells/ml) were cultured with vehicle control (ethanol), $1,25(OH)_2D_3$, retinoid (9cRA, ATRA, Am80, or HX630), or their combination for 24, 48, or 72 h.

Western blot analysis. Nuclear extracts from cells were prepared as described previously (20). The proteins were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a membrane for immunoblotting. Western blot analysis was performed using antibody to VDR and antibody to lamin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), visualized with an alkaline phosphatase conjugate substrate system as reported previously (21).

Reverse transcription and quantitative real-time polymerase chain reaction. Total RNA from cells was prepared by the acid guanidine thiocyanate-phenol/chloroform method (22). cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega Corporation, Madison, WI, USA). Real-time polymerase chain reaction (PCR) was performed on the ABI PRISM 7000 Sequence Detection System (Life Technologies Corporation, Rockville, MD, USA) with Power SYBR Green PCR Master Mix (Life Technologies Corporation). Primers were as follows: *CYP24A1* (GenBank accession no. NM_009996), 5'-TGG AGA CGA CCG CAA ACA G-3' and 5'-AGG CAG CAC GCT CTG GAT T-3'; *CAMP* (GenBank accession no. NM_009921), 5'-GGC CGC TGA TTC TTT TGA C-3' and 5'-CAC CAA TCT TCT CCC CAC CTT-3'; *VDR* (GenBank accession no. NM_009504), 5'-CCG CCA GAC CAG AGT TCT TTT-3' and 5'-AGG CAC ATT CCG GTC AAA GTC-3'. For relative mRNA expression, the mRNA values were normalized to the mRNA levels of β-actin as reported previously (17).

Statistics. All quantitative data were analyzed by one-way factorial ANOVA followed by Tukey's *post hoc* test using Prism 6 (Graphed Software, La Jolla, CA, USA).

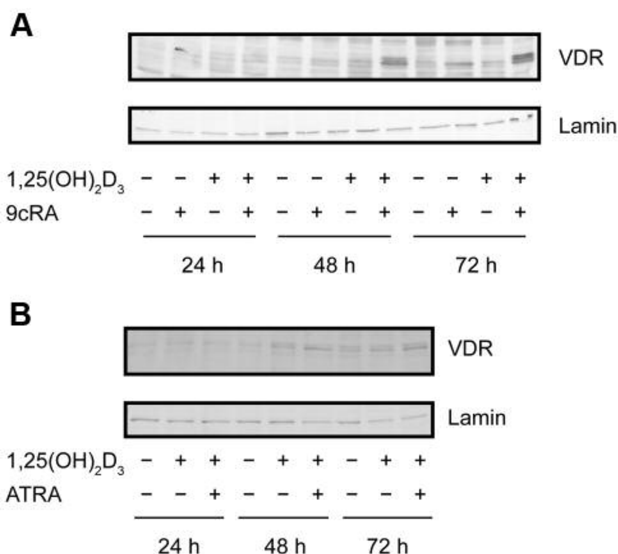

Figure 1. *Nuclear vitamin D receptor (VDR) expression in THP-1 cells treated with 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) plus retinoic acid. A: Effect of combined treatment with $1,25(OH)_2D_3$ and 9-cis retinoic acid (9cRA). B: Effect of combined treatment with $1,25(OH)_2D_3$ and all-trans retinoic acid (ATRA). Cells were treated with 100 nM $1,25(OH)_2D_3$ with/without 100 nM 9cRA or ATRA for 24, 48 or 72 h. Nuclear proteins were subjected to western blotting for VDR and lamin.*

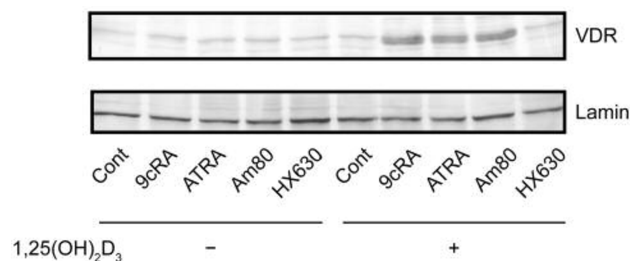

Figure 2. *Effect of selective retinoic acid receptor (RAR) and retinoid X receptor (RXR) ligands on 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-induced nuclear vitamin D receptor (VDR) expression in THP-1 cells. Cells were treated with 100 nM $1,25(OH)_2D_3$ with/without 30 nM 9-cis retinoic acid (9cRA), all-trans retinoic acid (ATRA), Am80 (synthetic RAR agonist), or HX630 (synthetic RXR agonist) for 72 h. Western blotting was performed for VDR and lamin.*

Results

Previous studies have shown that 9cRA is more potent than ATRA in inducing differentiation of monocytic leukemia cells (15-17, 23). We previously reported that $1,25(OH)_2D_3$ treatment increases nuclear VDR expression at 24 hours but levels decrease at 48 h in myeloid leukemia HL60 cells (21). We examined the effect of $1,25(OH)_2D_3$ with/without 9cRA

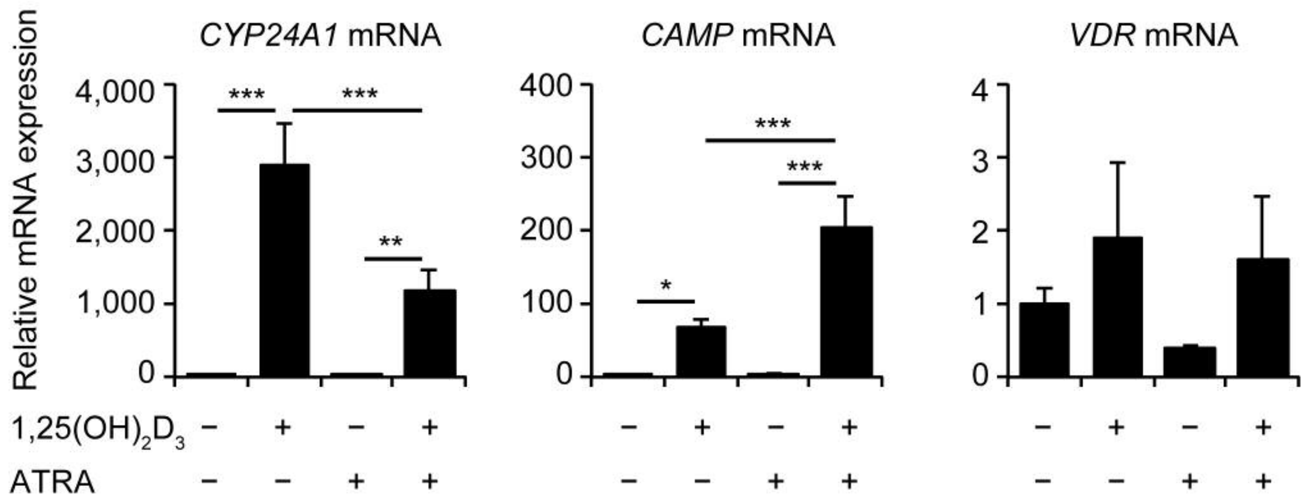

Figure 3. *Effect of 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$) plus all-trans retinoic acid (ATRA) on mRNA expression of vitamin D 24-hydroxylase (CYP24A1), cathelicidin antimicrobial peptide (CAMP) and vitamin D receptor (VDR). Cells were treated with 100 nM 1,25(OH)$_2$D$_3$ with/without 30 nM ATRA for 72 h. Expression of VDR target genes (CYP24A1 and CAMP) and VDR was evaluated with reverse transcription and quantitative real-time polymerase chain reaction. *p<0.05, **p<0.01 and ***p<0.001.*

on nuclear VDR expression in THP-1 monocytic leukemia cells. Similar to the previous report (21), 1,25(OH)$_2$D$_3$ treatment increased nuclear VDR expression at 48 h (Figure 1A). Interestingly, combined treatment with 1,25(OH)$_2$D$_3$ and 9cRA markedly increased VDR expression, while 9cRA alone had a weak effect. Nuclear VDR expression decreased at 72 hours in cells treated with 1,25(OH)$_2$D$_3$, but was maintained at a high level with the combination of 1,25(OH)$_2$D$_3$ plus 9cRA. A similar effect on nuclear VDR expression was observed with the combination of 1,25(OH)$_2$D$_3$ and ATRA (Figure 1B).

9cRA is a ligand for both RAR and RXR. Although ATRA is a ligand for RAR not for RXR, cells treated with ATRA have RXR activity after conversion to 9cRA (24, 25). In order to clarify which receptor is involved in increased nuclear VDR expression, we compared the effects of synthetic RAR agonist (Am80) and RXR agonist (HX630) in combination with 1,25(OH)$_2$D$_3$. In the absence of 1,25(OH)$_2$D$_3$, 9cRA, ATRA and Am80 slightly increased VDR expression (Figure 2). Combined treatment with 1,25(OH)$_2$D$_3$ and these retinoids effectively increased nuclear VDR protein levels. The RXR agonist HX630 was not effective in combination with 1,25(OH)$_2$D$_3$. Cytosolic VDR protein levels were not changed under any of the treatment conditions (data not shown). Thus, activation of RAR, not RXR, is involved in increased nuclear VDR expression.

Finally, we examined whether increased VDR expression affects VDR target-gene expression in cells treated with 1,25(OH)$_2$D$_3$ plus retinoic acid. We previously reported that the combination of 1,25(OH)$_2$D$_3$ and RAR ligand effectively induces mRNA expression of CD14, which is a VDR target gene and is a marker of monocytic differentiation (17). We examined the expression of other VDR target genes, *CYP24A1* and *CAMP*, which have a consensus direct repeat 3 element in the promoters. ATRA increased *CAMP* expression but reduced *CYP24A1* expression in cells treated with 1,25(OH)$_2$D$_3$, while ATRA alone had no effect (Figure 3). The combined effect of 1,25(OH)$_2$D$_3$ and ATRA may be gene selective. 1,25(OH)$_2$D$_3$ tended to increase *VDR* mRNA levels but combined treatment with ATRA did not further significantly increase its expression (Figure 3).

Discussion

We showed that combined treatment with 1,25(OH)$_2$D$_3$ and RAR ligand effectively increases nuclear VDR protein expression and expression of the VDR target gene *CAMP* in human monocytic leukemia cells. RAR ligand alone did not increase mRNA or protein expression of VDR, consistent with previous reports (23, 26, 27). The combination of 1,25(OH)$_2$D$_3$ and RAR ligand increased nuclear VDR protein expression but not *VDR* mRNA expression, as reported previously (23). Thus, increased nuclear VDR expression is mediated by a translational or post-translational mechanism. The selective RAR agonist Am80 exhibited a similar effect to ATRA and 9cRA on enhanced VDR expression, while the selective RXR agonist HX630 did not, indicating that the effect of retinoic acid on nuclear VDR expression is not mediated by RXR activation in the VDR–RXR heterodimer. Because treatment with RAR

ligand alone was not effective, RAR activation does not directly induce mRNA or protein expression of VDR. The combination of 1,25$(OH)_2D_3$ and RAR ligand was not effective in increasing *VDR* mRNA level or cytosolic VDR protein level. A small proportion of VDR may translocate from the cytosol to the nucleus in cells, and the change of cytosolic VDR expression may be difficult to detect. Nuclear VDR expression is regulated by several mechanisms, including nuclear import and export (8, 28) and protein degradation (21, 29). Protein modification, including phosphorylation, is also involved in VDR expression (21, 30). RAR signaling may increase nuclear VDR expression by enhancing its import or reducing its export or degradation. Further studies are needed to elucidate the detailed mechanism.

Combined treatment with 1,25$(OH)_2D_3$ and ATRA increased *CAMP* mRNA expression. Vitamin D signaling plays an important role in innate immune responses in monocytes and keratinocytes through the VDR-dependent induction of *CAMP* (31). Vitamin A is also essential in immune homeostasis, particularly in gut mucosal immunity (32). RAR ligands, ATRA and Am80, are used in the treatment of acute promyelocytic leukemia (33). Since application of 1,25$(OH)_2D_3$ and its derivatives in the treatment of non-calcemic diseases, such as leukemia, autoimmune/inflammatory disease, and infection, has a risk of hypercalcemia, a principal physiological effect of vitamin D, the combination of VDR ligand with other drugs, such as retinoic acid, may be useful in reducing adverse effects (34). ATRA treatment increased *CAMP* expression but not *CYP24A1* expression, indicating a gene-selective effect of RAR signaling on VDR target-gene expression. Therefore, combined treatment with VDR and RAR ligands may be useful for enhancement of selective VDR function, specifically in the immune response.

Conflicts of Interests

The Authors declare no conflicts of interest in regard to this study.

Acknowledgements

The Authors thank members of the Makishima laboratory for technical assistance and Dr. Andrew I. Shulman for editorial assistance. This work was supported in part by "Open Research Center" Project for Private Universities subsidized by MEXT (2005-2009) and "Strategic Research Base Development" program for Private Universities subsidized by MEXT (2010-2014).

References

1 Nagpal S, Na S and Rathnachalam R: Noncalcemic actions of vitamin D receptor ligands. Endocr Rev *26*: 662-687, 2005.

2 Plum LA and DeLuca HF: Vitamin D, disease and therapeutic opportunities. Nat Rev Drug Discov *9*: 941-955, 2010.

3 van Leeuwen JPTM, van Driel M, Feldman D and Munoz A: Vitamin D: cancer and differentiation. *In*: Vitamin D. Feldman D, Pike JW and Adams JS (eds.). Waltham, MA, USA: Academic Press, pp. 1591-1624, 2011.

4 Honma Y, Hozumi M, Abe E, Konno K, Fukushima M, Hata S, Nishii Y, DeLuca HF and Suda T: 1α,25-Dihydroxyvitamin D_3 and 1α-hydroxyvitamin D3 prolong survival time of mice inoculated with myeloid leukemia cells. Proc Natl Acad Sci USA *80*: 201-204, 1983.

5 Okamoto R and Koeffler HP: Hematological malignancy. *In*: Vitamin D. Feldman D, Pike JW and Adams JS (eds.). Waltham, MA, USA: Academic Press, pp. 1731-1750, 2011.

6 Yamada S and Makishima M: Structure–activity relationship of nonsecosteroidal vitamin D receptor modulators. Trends Pharmacol Sci *35*: 324-337, 2014.

7 Gocek E, Kiekbinski M and Marcinkowska E: Activation of intracellular signaling pathways is necessary for an increase in VDR expression and its nuclear translocation. FEBS Lett *581*: 1751-1757, 2007.

8 Miyauchi Y, Michigami T, Sakaguchi N, Sekimoto T, Yoneda Y, Pike JW, Yamagata M and Ozono K: Importin 4 is responsible for ligand-independent nuclear translocation of vitamin D receptor. J Biol Chem *280*: 40901-40908, 2005.

9 Haussler MR, Whitfield GK, Kaneko I, Haussler CA, Hsieh D, Hsieh JC and Jurutka PW: Molecular mechanisms of vitamin D action. Calcif Tissue Int *92*: 77-98, 2013.

10 Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, Yoshiki S and Suda T: Differentiation of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA *78*: 4990-4994, 1981.

11 Honma Y, Takenaga K, Kasukabe T and Hozumi M: Induction of differentiation of cultured human promyelocytic leukemia cells by retinoids. Biochem Biophys Res Commun *95*: 507-512, 1980.

12 Breitman TR, Selonick SE and Collins SJ: Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA *77*: 2936-2940, 1980.

13 Olsson IL and Breitman TR: Induction of differentiation of the human histiocytic lymphoma cell line U-937 by retinoic acid and cyclic adenosine 3':5'-monophosphate-inducing agents. Cancer Res *42*: 3924-3927, 1982.

14 Mehta K and Lopez-Berestein G: Expression of tissue transglutaminase in cultured monocytic leukemia (THP-1) cells during differentiation. Cancer Res *46*: 1388-1394, 1986.

15 Defacque H, Sevilla C, Piquemal D, Rochette-Egly C, Marti J and Commes T: Potentiation of VD-induced monocytic leukemia cell differentiation by retinoids involves both RAR and RXR signaling pathways. Leukemia *11*: 221-227, 1997.

16 Makishima M, Shudo K and Honma Y: Greater synergism of retinoic acid receptor (RAR) agonists with vitamin D3 than that of retinoid X receptor (RXR) agonists with regard to growth inhibition and differentiation induction in monoblastic leukemia cells. Biochem Pharmacol *57*: 521-529, 1999.

17 Takahashi H, Hatta Y, Iriyama N, Hasegawa Y, Uchida H, Nakagawa M, Makishima M, Takeuchi J and Takei M: Induced differentiation of human myeloid leukemia cells into M2 macrophages by combined treatment with retinoic acid and 1α,25-dihydroxyvitamin D3. PLoS ONE *9*: e113722, 2014.

18 Makishima M: Cholesterol and bile acid metabolisms regulated by nuclear receptors. J Pharmacol Sci *94*: 23P-23P, 2004.

19 Shulman AI, Larson C, Mangelsdorf DJ and Ranganathan R: Structural determinants of allosteric ligand activation in RXR heterodimers. Cell *116*: 417-429, 2004.
20 Schreiber E, Matthias P, Muller MM and Schaffner W: Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res *17*: 6419, 1989.
21 Amano Y, Cho Y, Matsunawa M, Komiyama K and Makishima M: Increased nuclear expression and transactivation of vitamin D receptor by the cardiotonic steroid bufalin in human myeloid leukemia cells. J Steroid Biochem Mol Biol *114*: 144-151, 2009.
22 Tavangar K, Hoffman AR and Kraemer FB: A micromethod for the isolation of total RNA from adipose tissue. Anal Biochem *186*: 60-63, 1990.
23 Nakajima H, Kizaki M, Ueno H, Muto A, Takayama N, Matsushita H, Sonoda A and Ikeda Y: All-*trans* and 9-*cis* retinoic acid enhance 1,25-dihydroxyvitamin D_3-induced monocytic differentiation of U937 cells. Leuk Res *20*: 665-676, 1996.
24 Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM and Thaller C: 9-*cis* retinoic acid is a high affinity ligand for the retinoid X receptor. Cell *68*: 397-406, 1992.
25 Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A and Gripppo JF: 9-*Cis* retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature *355*: 359-361, 1992.
26 Gocek E, Marchwicka A, Baurska H, Chrobak A and Marcinkowska E: Opposite regulation of vitamin D receptor by ATRA in AML cells susceptible and resistant to vitamin D-induced differentiation. J Steroid Biochem Mol Biol *132*: 220-226, 2012.
27 Folgueira MAAK, Federico MHH, Katayama MLH, Silva MRP and Brentani MM: Expression of vitamin D receptor (VDR) in HL-60 cells is differentially regulated during the process of differentiation induced by phorbol ester, retinoic acid or interferon-gamma. J Steroid Biochem Mol Biol *66*: 193-201, 1998.
28 Yasmin R, Williams RM, Xu M and Noy N: Nuclear import of the retinoid X receptor, the vitamin D receptor, and their mutual heterodimer. J Biol Chem *280*: 40152-40160, 2005.
29 Malloy PJ and Feldman D: Inactivation of the human vitamin D receptor by caspase-3. Endocrinology *150*: 679-686, 2009.
30 Gupta K, Stefan T, Ignatz-Hoover J, Moreton S, Parizher G, Saunthararajah Y and Wald DN: GSK-3 inhibition sensitizes acute myeloid leukemia cells to 1,25D-mediated differentiation. Cancer Res *76*: 2743-2753, 2016.
31 Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C, Kamen DL, Wagner M, Bals R, Steinmeyer A, Zugel U, Gallo RL, Eisenberg D, Hewison M, Hollis BW, Adams JS, Bloom BR and Modlin RL: Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science *311*: 1770-1773, 2006.
32 Mora JR, Iwata M and von Andrian UH: Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol *8*: 685-698, 2008.
33 Tomita A, Kiyoi H and Naoe T: Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) in acute promyelocytic leukemia. Int J Hematol *97*: 717-725, 2013.
34 Takada I and Makishima M: Therapeutic application of vitamin D receptor ligands: an updated patent review. Expert Opin Ther Pat *25*: 1373-1383, 2015.

Received September 26, 2016
Revised October 5, 2016
Accepted October 10, 2016