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1α,25-Dihydroxyvitamin D_3-26,23-lactam analogues function as vitamin D receptor antagonists in human and rodent cells

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Abstract

(23*S*,25*S*)-N-Benzyl-1α,25-dihydroxyvitamin D_3-26,23-lactam ((23*S*,25*S*)-N-benzyl- 1α,25-$(OH)_2D_3$-26,23-lactam, (23*S*,25S)-DLAM-1P) antagonizes nuclear vitamin D receptor (VDR)-mediated differentiation of human promyelocytic leukemia (HL-60) cells [Bioorg. Med. Chem. Lett. 14, 2579–2583(2004)]. To enhance its VDR antagonistic actions, we synthesized multiple analogues of 1α,25-$(OH)_2D_3$-26,23-lactam. Among these analogues, (23*S*,25*S*)-N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam, ((23*S*,25*S*)- DLAM-2P) had the strongest VDR binding affinity, which was 3 times higher than that of (23*S*,25*S*)-DLAM-1P. The 1α,25-$(OH)_2D_3$-26,23-lactam analogues never induced HL-60 cell differentiation even at 10^{-6}M, but (23*S*,25S)-DLAM-1P and (23*S*,25*S*)-DLAM-2P significantly and dose-dependently inhibited HL-60 differentiation induced by 10^{-8}M 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$). These compounds also inhibited human and mouse cultures of osteoclast formation by marrow cells treated with 1α,25-$(OH)_2D_3$. Moreover, the 1α,25-$(OH)_2D_3$-26,23-lactam analogues minimally induced 25-hydroxy- vitamin D_3-24-hydroxylase gene expression in HL-60 cells and human and mouse osteoblastic cells, but 10^{-6}M (23*S*,25*S*)-DLAM-1P or (23*S*,25*S*)-DLAM-2P significantly blocked 24-hydroxylase gene expression induced by 10^{-8}M 1α,25-$(OH)_2D_3$. (23*S*,25*S*)- DLAM-2P was 5 to 12 times more potent as a Vitamin D antagonist than (23*S*,25*S*)-DLAM-1P in HL-60 cells, human and mouse bone marrow cultures. These results demonstrate that (23*S*,25*S*)-DLAM-1P and (23*S*,25*S*)-DLAM-2P antagonize HL-60 cell differentiation and osteoclast formation by human and mouse osteoclast precursors induced by 1α, 25-$(OH)_2D_3$ through blocking VDR-mediated gene transcription. In contrast, (23*S*)-25-deoxy-1α-hydroxyvitamin D_3-26,23-lactone, which only blocks human VDR, these vitamin D antagonists can block VDR in human cells and rodent cells.

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Keywords

Vitamin D receptor antagonist; 1α; 25-dihydroxyvitamin D_3-26,23-lactam analogues; HL-60 cell differentiation; osteoclast formation; gene expression

Introduction

To date, a large number of vitamin D_3 analogues have been synthesized; however, almost all of these compounds are vitamin D agonists [1]. 1β,25-Dihydroxyvitamin D_3 (1β,25-$(OH)_2D_3$) is a well known vitamin D antagonist, which acts through rapid nongenomic actions via a putative membrane vitamin D receptor (VDR). It does not act as a vitamin D antagonist for nuclear VDR-mediated gene transcription induced by 1α,25-dihydroxyvitamin D_3 (1β,25-$(OH)_2D_3$) [2,3]. Currently, only three types of 1α,25-$(OH)_2D_3$ analogues, which showed nuclear VDR antagonistic actions, have been described. These are the (23*S*)- and (23*R*)-25-dehydro-1α-hydroxyvitamin D_3-26,23- lactones (TEI-9647 and TEI-9648, respectively) [4–6], the 25-carboxylic ester derivatives of 1α,25-$(OH)_2D_3$ (ZK159222 and ZK168281) [7,8], and the (23*S*,25*S*)-N-benzyl- 1α,25-dihydroxyvitamin D_3-26,23-lactam ((23*S*,25*S*)-N-benzyl-1α,25-$(OH)_2D_3$-26,23- lactam, (23*S*,25*S*)-DLAM-1P) [9].

Previously, we reported that a 1α,25-dihydroxyvitamin D_3-26,23-lactone (1α,25-$(OH)_2D_3$-26,23-lactone) analogue, TEI-9647, inhibits monocyte differentiation of human promyelocytic leukemia (HL-60) cells, and blocks osteoclast (OCL) formation by human bone marrow cells treated with 1α,25-$(OH)_2D_3$ [4–6,10,11]. TEI-9647 shows significant vitamin D antagonistic activity in terms of 25-hydroxyvitamin D_3-24- hydroxylase (25-OH-D_3-24-hydroxylase) and $p21^{waf1/cip1}$ gene expression induced by 1α,25-$(OH)_2D_3$ in HL-60 cells and human osteosarcoma cells (SaOS-2 and MG-63 cells) [4,6,12]. Moreover, we demonstrated that TEI-9647 prevents heterodimer complex formation between the VDR and retinoid X receptor (RXR), and recruitment of steroid receptor coactivator-1 (SRC-1) to VDR [13].

Very recently, both Carlberg and our group reported that TEI-9647 displays vitamin D antagonistic actions in human cells but has vitamin D agonistic actions in rodent cells [14, 15]. The vitamin D antagonistic actions of TEI-9647 depend on the primary structure of the carboxyl-terminal region of the VDR. The C-terminal regions (the last 67 residues) of the ligand binding domain of human and rodent VDR are highly conserved but differ from each other at the L378, C403 and C410 of human and the corresponding residues of rat VDR. We demonstrated that interaction between the exo-methylene structure and the cysteine residues at 403 and/or 410 in human VDR is critical for the antagonistic activity of TEI-9647 [14,15]. Unlike TEI-9647, ZK159222 and ZK168281, which are well-known VDR antagonists, exhibit their antagonistic effects regardless of the species of VDR [7,8,14–16].

Kato et al. recently reported that another type of vitamin D antagonist, (23*S*,25*S*)-DLAM-1P, which inhibits HL-60 cell differentiation induced by 1α,25-$(OH)_2D_3$, but its vitamin D antagonistic activity seems to be extremely weak compared to TEI-9647 [9]. Therefore, we synthesized multiple analogues of the 1α,25-$(OH)_2D_3$-26,23-lactam in order to enhance its vitamin D antagonistic activity, and evaluated these analogues in human and rodent cells. In this paper, we demonstrate that the (23*S*,25*S*)- N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam ((23*S*,25*S*)-DLAM-2P) was a highly potent vitamin D antagonist in human and rodent cells.

Materials and Methods

Chemicals

1α,25-$(OH)_2D_3$, (23*S*,25*R*)-1α,25-$(OH)_2D_3$-26,23-lactone, TEI-9647 and 1α,25-$(OH)_2D_3$-26,23-lactam analogues were synthesized in our laboratory as described previously [9,17–20]; their structures are shown in Fig. 1. Each compound was dissolved in absolute ethanol. [26,27-methyl-^{3}H]1α,25-$(OH)_2D_3$ (specific activity, 6.623TBq/mmol) was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Fetal bovine serum (FBS) was purchased from GIBCO-BRL Life Technologies, Inc. (Grand Island, NY). Nitro blue tetrazolium (NBT) was bought from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Kernechrot solution was obtained from Muto Pure Chemicals Co. Ltd. (Tokyo, Japan). All other chemicals and media were purchased from Sigma Chemical Corporation (St. Louis, MO), unless otherwise noted.

Binding affinity for vitamin D receptor

Competitive receptor binding assays for 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$-26,23-lactam analogues were performed using VDR from chick intestine as described previously [20]. In this assay, 0.1 mg protein/ml of chick VDR and 18,000 dpm of [26,27-methyl-^{3}H]1α,25-$(OH)_2D_3$ (specific activity, 6.623TBq/mmol, 18.8pg) were used.

Cell and culture

HL-60 cells and a human osteoblastic cell line (HOS cells) were obtained from the Japanese Cancer Research Resources Bank. Cells were passaged twice a week to maintain them in an exponential proliferating phase. RPMI-1640 (GIBCO BRL, Life Technologies, Inc. Rockville, MO) containing 10% heat inactivated FBS (Bioserum, Lot No.: 01307-01) for the HL-60 cells, and Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co. Tokyo, Japan) supplemented with 10% dextran-coated charcoal-stripped FBS (JRH Bioscience, Dexton, KS), penicillin and streptomycin for the HOS cells, were used as culture media. Cell counts were determined with a hemocytometer, and the viability of cells was tested by trypan-blue dye exclusion.

Assay for HL-60 cell differentiation

NBT reducing activity was used as a marker of cell differentiation. HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated FBS. Exponentially proliferating cells were collected, suspended in fresh media and seeded in culture vessels. 24-Well culture plates (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ) were used. Cell concentration at seeding was adjusted to 2×10^4 cells/ml and seeding volume was 1 ml/well. 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$-26,23-lactam analogues dissolved in ethanol were added to the culture media at 0.1% volume and the cells cultured for 96 hrs at 37°C in a humidified atmosphere of 5% CO_2-air without media change. The same amount of vehicle was added to the control cultures. NBT reducing assays were performed as described previously [4] according to the method of Collins et al. [21].

Subjects and cell preparation

Bone marrow cells aspirated under 2% xylocaine anesthesia from the iliac crest of healthy normal volunteers into heparinized alpha-Minimal Essential Media (α-MEM) containing 5% FBS, as previously described [22]. Bone marrow mononuclear cells were then isolated by separation on Hypaque-Ficoll gradients (density 1.077g/ml), centrifuged at 400 g for 30 minutes and then washed three times with α-MEM, as described previously [22]. The Institutional Review Board of the University of Pittsburgh approved these studies.

Measles virus nucleocapsid protein (MVNP) gene transduction of human bone marrow cell

MVNP-transduced human marrow cells were used for these assays because they are hyper-responsive to 1α,25$(OH)_2D_3$ and form osteoclasts at concentrations of 1α,25$(OH)_2D_3$ that are 1–2 logs lower than normal cells. These cells were used as a more sensitive assay for the potential agonistic actions of the lactam analogues. Human bone marrow mononuclear cells were cultured for 2 days in α-MEM containing 10% FBS that contained 10ng/ml each of IL-3, IL-6 and stem cell factor (SCF) (Amgen Immunex Research and Development Corporation; Seattle, WA). The bone marrow cells were then cultured for an additional 48 hours at 37°C in a humidified atmosphere of 5% CO_2-air at a density of 1 to 2 × 105 cells/ml with supernatant (10% v/v) containing the MVNP vector [23]. Cultures were supplemented with 4μg/ml of polybrene, 20ng/ml of IL-3, 50ng/ml of IL-6 and 100ng/ml of SCF as described previously [23]. MVNP-transduced cells were suspended at 10^6 cells/ml in α-MEM containing 1.2% methylcellulose, 30% FBS, 1% deionized bovine serum albumin (BSA) and 100pg/ml recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) (Amgen Immunex Research and Development Corporation; Seattle, WA) with 250μg/ml of G418. Transduced cells were plated in a volume of 1 ml in 35-mm culture dishes (Corning; New York, NY) and incubated at 37°C in a humidified atmosphere of 5% CO_2-air for 7 days, for isolation of G418 resistance colony-forming unit-granulocyte macrophage (CFU-GM) cells. The G418-resistant colonies were individually collected, using finally drawn pipettes, for use in OCL formation assays employing MVNP-transduced CFU-GM cells.

Osteoclast formation induced by 1α,25-$(OH)_2D_3$

OCL formation from MVNP- transduced CFU-GM cells induced by 1α,25-$(OH)_2D_3$ was performed as described previously [11,23]. Briefly, MVNP-transduced CFU-GM cells (10^6 cells/ml) were dispersed into α-MEM containing 20% horse serum, and were seeded in 96-well multi-plates (Becton Dickinson Labware, Frankin Lakes, NJ) at 100μl/well. 10^{-9}M 1α, 25-$(OH)_2D_3$, TEI-9647 (10^{-9}M to 10^{-6}M), 1α,25-$(OH)_2D_3$-26,23-lactam analogues (10^{-8}M to 10^{-6}M), alone or in combination with 1α,25-$(OH)_2D_3$ (10^{-9}M) were added into a well. Half of the media was replaced two times a week, and the cultures were continued for 3 weeks at 37°C in an incubator of 5% CO_2-air. The OCL that formed were then fixed with 2% formaldehyde and tested for cross-reactivity with the monoclonal antibody 23C6, which recognized the osteoclast vitronectin receptor (generously provided by Dr. Michael Horton, Rayne Institute, Bone and Mineral Center, London, United Kingdom), using a Vectastatin-ABC-AP kit (Vector Laboratories, Burlingame, CA), as described previously [22,23]. Cells that cross-reacted with the 23C6 antibody and had 3 or more nuclei were scored as OCL using an inverted microscope.

Osteoclast formation by mouse bone marrow cells treated with 1α,25-$(OH)_2D_3$

OCL formation by mouse bone marrow cells treated with 1α,25-$(OH)_2D_3$ was performed as described previously [24]. Briefly, six-week-old mice (ICR strain) were used for all experiments. The femora were disarticulated, soft tissues were dissected away, and then both ends of each bone were removed. The bone marrow cells were flushed out with a syringe containing α-MEM, and a single cell suspension was prepared by pipetting. One hundred microliter of unfractionated bone marrow cells (10^6 cells/ml) was plated in each well of 96-well multi-plates (Becton Dickinson Labware, Franklin Lakes, NJ) and cultured at 37°C in humidified 5% CO_2-air incubator. The media consisted of αMEM containing 10% FBS that had been inactivated at 65°C for 30 min. After 1 day, the cells were transferred to media containing various concentrations of vitamin D analogues, and thereafter the cultures were fed every 3 days by replacing half the media. After 8 days of culture, the cells were fixed with 1% formaldehyde and stained with tartrate resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma Chemical Corporation, St. Louis, MO). The TRAP-positive

multinucleated cells (> 3 nuclei/cell) were scored as OCL using an inverted microscope. The animal care and the experimental protocols were approved by the Animal Experimentation Ethics Committee of the University of Pittsburgh and the VA Pittsburgh Healthcare System.

25-Hydroxyvitamin D_3-24-hydroxylase (25-OH-D_3-24-hydroxylase) gene expression

To investigate the 25-OH-D_3-24-hydroxylase gene expression induced by 1α,25-$(OH)_2D_3$ or 1α,25-$(OH)_2D_3$-26,23-lactam analogues, HL-60 cells (10^5 cells/ml medium in a 35mm-diameter dish, 3 ml) were incubated at 37°C in a RPMI-1640 media containing 10% heat inactivated FBS with 10^{-8}M 1α,25-$(OH)_2D_3$, or 10^{-6}M 1α,25-$(OH)_2D_3$- 26,23-lactam analogues alone, or in combination for 24 hrs. In the case of HOS cells and primary mouse osteoblastic cells, culture media were changed to DMEM and αMEM, respectively. After incubation, total RNA was extracted by the acid guanidium thiocyanate-phenol method (Isogen, Nippon Gene, Tokyo, Japan). cDNA was synthesized from 1μg of total RNA using random hexamers and reverse transcriptase (GeneAmp; RNA PCR Core kit, Applied Biosystems, Foster City, CA) according to their manual. For gene amplification of cDNAs for 25-OH-D_3-24-hydroxylase and β-actin (RNA 50ng and 12.5ng, respectively), were added to 100 pmoles of each PCR primer and 2.5 units Taq polymerase (Applied Biosystems, Foster City, CA). Both samples (total 50μl reaction volume) were subjected to PCR amplification in a programmed thermal cycler. For human 25-OH-D_3-24-hydroxylase amplification, the PCR primers were 5′ to 3′ GGGAAGTGATGAAGCTGGAC (898–917) and TCATAGATTCTTTCAGACAGG (1526–1546). For mouse 25-OH-D_3-24-hydroxylase amplification, the PCR primer was 5′ to 3′ ATTACCTGAGAATCAGAGGCCACG (1049–1068) and GCCAAATGCAGTTT AAGCTCTGCT (1524–1543). PCR cycles were as follows; 60 sec at 94°C for denaturation, 60 sec at 60°C for annealing, 90 sec at 72°C for extension, 26 cycles. For β-actin gene amplification, the PCR primers were 5′ to 3′ ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA. PCR cycles were as follows; 60 sec at 94°C for denaturation, 60 sec at 60°C for annealing, 90 sec at 72°C for extension, 25 cycles. PCR products were analyzed by 2% agarose gel electrophoresis (648 bp product and 495 bp product were obtained in human 25-OH-D_3-24-hydroxylase gene and mouse 25-OH-D_3-24-hydroxylase gene PCR, and 280bp product was obtained in β-actin gene PCR).

Reporter gene assay

The promoter region of the human 25-OH-D_3-24-hydroxylase gene (−186 to −5), which contains two vitamin D responsive elements (VDRE; a gift from Dr. H. Eguchi, Teijin Institute for Bio-Medical Research, Tokyo, Japan), was cloned into a luciferase reporter vector pGL3-Basic Vector (Promega, Madison, WI) as described previously [26]. This plasmid construct was co-transfected with the β-galactosidase expression plasmid into NIH3T3 cells transduced with MVNP using the DMRIE-C Regent (Invitrogen Life Technology). Sixteen hours after transfection, vehicle (0.1% ethanol) or 10^{-9}M 1α,25-$(OH)_2D_3$, or 10^{-6}M (23*S*,25*S*)-DLAM-2P or combination of 10^{-9}M 1α,25-$(OH)_2D_3$ and (23*S*,25*S*)-DLAM-2P (10^{-9}M to 10^{-6}M) were added. Twenty-four hours later, the cells were harvested and lysed in the cell lysate solution provided with the luciferase assay kit (Promega, Madison, WI). The luciferase activities of the cell lysates were measured with the luciferase assay kit according to the manufacturer's instructions and were standardized by comparing the galactosidase activities of the same cell lysates as determined with a β-galactosidase enzyme assay system (Promega, Madison, WI).

Statistical analysis

Each data point is shown mean ± S.D. of triplicate or quadruplicate determinations. Results are representative of two or three independent experiments. The data were analyzed using a two-tailed Student's *t*-test, with $p < 0.05$ considered significant.

Results

Figure 1 indicates the structures of 1α,25-$(OH)_2D_3$-26,23-lactam analogues, which are very similar with those of the 1α,25-$(OH)_2D_3$-26,23-lactone analogues. In the lactam structure, one nitrogen atom replaces an oxygen atom in the lactone ring. N-Benzyl- and N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam have 2 asymmetric carbons at C23 and C25. Therefore, they have four diastereoisomers, respectively.

The binding affinities of the 1α,25-$(OH)_2D_3$-26,23-lactam analogues to chick VDR are shown in Table 1. The VDR binding affinities of (23*S*,25*S*)-DLAM-1P and (23*S*,25*S*)-DLAM-2P, which have 23*S* and 25*S* configurations, were 36.4 and 12.6 times weaker than that of 1α,25-$(OH)_2D_3$, respectively. The VDR binding affinity of (23*S*,25*S*)-DLAM-2P was about 3 times higher than that of (23*S*,25*S*)-DLAM-1P. The other stereoisomers of 1α,25-$(OH)_2D_3$-26,23-lactam had only a weak VDR binding affinity compared with (23*S*,25*S*)-DLAM-1P or (23*S*, 25*S*)-DLAM-2P, respectively.

Previously, Kato *et al.* reported that (23*S*,25*S*)-DLAM-1P never induced HL-60 cell differentiation even after treatment at 10^{-6}M, but that 10^{-6}M (23*S*,25*S*)-DLAM-1P significantly inhibited HL-60 differentiation induced by 10^{-8}M 1α,25-$(OH)_2D_3$ [9]. We examined the inhibitory actions of 1α,25-$(OH)_2D_3$-26,23-lactam analogues on HL-60 cell differentiation induced by 1α,25-$(OH)_2D_3$ in more detail, using NBT reducing activity as a cell differentiation marker. Concentrations of 10^{-9}M to 10^{-7}M 1α,25-$(OH)_2D_3$ dose-dependently induced differentiation of HL-60 cells during the 96 hrs culture period (data not shown). 10^{-8}M 1α,25-$(OH)_2D_3$ differentiated >70% of the cells into NBT-reducing activity positive cells, however all diastereoisomers of N-benzyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues and N-phenetyl-1α,25-$(OH)_2D_3$- 26,23-lactam analogues did not induce any cell differentiation even at concentrations of 10^{-6}M or 2×10^{-6}M (Figure 2A and 3A). However, (23*S*,25*S*)-DLAM-1P, (23*S*,25*S*)-DLAM-2P and (23*S*,25*R*)-DLAM-2P dose-dependently inhibited HL-60 cell differentiation induced by 10^{-8}M 1α,25-$(OH)_2D_3$ (Figures 2B and 3B). The (23*S*,25*S*)-DLAM-1P and (23*S*,25*R*)-DLAM-2P showed a similar dose-response curve, but their suppressive effects were consistently weaker than that of (23*S*,25*S*)-DLAM-2P (Figures 2B and 3B). On the contrary, another type of vitamin D antagonist, TEI-9647 dose-dependently inhibited HL-60 cell differentiation induced by 10^{-8}M 1α,25-$(OH)_2D_3$ (Figures 2B and 3B). Based on the HL-60 cell differentiation assays, the vitamin D antagonistic actions of (23*S*,25*S*)-DLAM-1P, (23*S*,25*S*)-DLAM-2P and (23*S*,25*R*)- DLAM-2P were estimated to be about 1.32, 8.33 and 0.75% of TEI-9647 activity, respectively.

We previously demonstrated that TEI-9647 was a vitamin D antagonist in human cells but had weak vitamin D agonistic actions in rodent cells [14]. Therefore, we investigated whether the 1α,25-$(OH)_2D_3$-26,23-lactam analogues were vitamin D antagonists in both human cells and rodent cells, using OCL formation assays with human and mouse bone marrow cells. We confirmed our previous results that 1α,25-$(OH)_2D_3$ induced OCL formation through VDR-mediated transcription in marrow cultures from MVNP-transduced normal OCL precursors (MVNP-transduced CFU-GM cells) [11,25,26]. Using MVNP-transduced CFU-GM cells, 10^{-11}M to 10^{-9}M 1α,25-$(OH)_2D_3$ dose-dependently stimulated OCL formation. OCL formation in these cultures was significantly increased above control levels at 10^{-11}M 1α,25-$(OH)_2D_3$ and reached maximum levels at 10^{-9}M 1α,25-$(OH)_2D_3$ (data not shown). TEI-9647 did not stimulate OCL formation in any of the cultures, even at high concentrations (10^{-6}M). In contrast, TEI-9647 dose-dependently blocked OCL formation induced by 10^{-9}M 1α,25-$(OH)_2D_3$ (Figure 4A). All stereoisomers of N-benzyl-1α,25-$(OH)_2D_3$-26,23-lactam and N-phenetyl-1α,25-$(OH)_2$ D_3-26,23-lactam did not stimulate OCL formation in any of the cultures, even at 10^{-6}M. (23*S*,25*S*)-DLAM-1P and (23*S*,25*S*)-DLAM-2P dose-dependently blocked OCL formation induced by 10^{-9}M 1α,25-$(OH)_2D_3$. The OCL formation inhibitory action of

(23*S*,25*S*)-DLAM-2P was about 5 times greater than that of (23*S*,25*S*)-DLAM-1P (Figure 4A). Similarly, the other stereoisomers of (23*S*,25*S*)-DLAM-1P and (23*S*,25*S*)-DLAM-2P, (23*S*, 25*R*)-DLAM-1P and (23*S*,25*R*)- DLAM-2P dose-dependently blocked OCL formation induced by 10^{-9}M 1α,25-$(OH)_2D_3$. However, these activities were significantly weaker compared to those of (23*S*,25*S*)-DLAM-1P and (23*S*,25*S*)-DLAM-2P (Figure 4A).

We previously demonstrated that 10^{-9}M 1α,25-$(OH)_2D_3$ significantly stimulated OCL formation in mouse bone marrow cell cultures, and the number of OCL increased linearly up to 10^{-7}M [24]. In mouse bone marrow cell cultures, all of the compounds tested showed similar inhibitory action on OCL formation induced by 10^{-8}M 1α,25-$(OH)_2D_3$, except TEI-9647 (Figure 4B). The inhibitory action of TEI-9647 on OCL formation by normal mouse bone marrow cells treated with 10^{-8}M 1α,25-$(OH)_2D_3$ was lower as compared to human bone marrow cells (Table 2).

We then performed time-course and dose-response experiments to assess the antagonistic action of TEI-9647 and 1α,25-$(OH)_2D_3$-26,23-lactam analogues on VDR-mediated gene transcription using HL-60 cells, the human osteoblastic cell line, HOS cells, and primary mouse osteoblastic cells. We examined the time-course changes in 25-OH-D_3-24-hydroxylase mRNA induced by 10^{-8}M 1α,25-$(OH)_2D_3$ in HL-60 cells. The 25-OH-D_3-24-hydroxylase mRNA was minimally detectable after 4–8 hrs treatment with 1α,25-$(OH)_2D_3$, but was expressed at significant levels after 24–48 hrs. 10^{-9}M to 10^{-6}M 1α,25-$(OH)_2D_3$ dose-dependently induced 25-OH-D_3-24-hydroxylose mRNA, and maximum activity was observed at 10^{-7}M (data not shown). Even at 10^{-6}M concentration, neither TEI-9647 nor the 1α,25-$(OH)_2D_3$-26,23-lactam analogues by themselves induced 25-OH-D_3-24-hydroxylase gene expression (lanes 3 to 7 in Figure 5A), although the structural analogue of 1α,25-$(OH)_2D_3$-26,23-lactam, 10^{-6}M 1α,25-$(OH)_2D_3$-26,23-lactone, significantly induce it (lane 8 in Figure 5A). However, 10^{-6}M TEI-9647 and 10^{-6}M (23S,25S)-DLAM-2P almost completely suppressed the 25-OH-D_3-24-hydroxylase gene expression induced by 10^{-8}M 1α,25-$(OH)_2D_3$ in HL-60 cells (lanes 9 and 12 in Figure 5A). 10^{-6}M (23*S*,25*S*)-DLAM-1P weakly but significantly suppressed 25-OH-D_3-24-hydroxylase gene expression induced by 10^{-8}M 1α,25-$(OH)_2D_3$ (lane 10 in Figure 5A). The inhibitory action of (23*S*,25*S*)-DLAM-1P was much weaker than that of (23*S*,25*S*)-DLAM-2P. On the contrary, stereoisomers of (23*S*,25*S*)-DLAM-1P and (23*S*,25*S*)-DLAM-2P, (23*S*,25*R*)-DLAM-1P and (23*S*,25*R*)- DLAM-2P, never and weakly inhibited 25-OH-D_3-24-hydroxylase activity induced by 10^{-8}M 1α,25-$(OH)_2D_3$ even at 10^{-6}M, respectively (lanes 11 and 13 in Figure 5A). Moreover, 10^{-6}M 1α,25-$(OH)_2D_3$-26,23-lactone enhanced the effects of 10^{-8}M 1α,25-$(OH)_2D_3$ on 25-OH-D_3-24-hydroxylase activity (lane 14 in Figure 5A). Similar results were seen with HOS cells and mouse osteoblastic cells. However, 10^{-6}M (23*S*,25*R*)-DLAM-1P and (23*S*,25*R*)-DLAM-2P weakly but significantly blocked the 25-OH-D_3-24-hydroxylase gene expression induced by 10^{-8}M 1α,25-$(OH)_2D_3$ (lanes 11 and 13 in Figures 5B and 5C). In mouse osteoblastic cells, 10^{-6}M TEI-9647 did not inhibit 25-OH-D_3-24-hydroxylase gene expression induced by 10^{-8}M 1α,25-$(OH)_2D_3$ a result different from that obtained using human cells(lane 9 in Figure 5C), but 10^{-6}M (23*S*,25*S*)-DLAM-1P and (23*S*,25*S*)-DLAM-2P almost completely suppressed 25-OH-D_3-24-hydroxylase gene expression induced by 10^{-8}M 1α,25-$(OH)_2D_3$ (lanes 10 and 12 in Figure 5C). 1α,25-$(OH)_2D_3$-26,23-Lactone itself weakly but significantly induced 25-OH-D_3-24-hydroxylase mRNA (lane 8 in Figure 5).

Among 1α,25-$(OH)_2D_3$-26,23-lactam analogues, (23*S*,25*S*)-DLAM-2P showed the strongest inhibitory action of gene expression induced by 1α,25-$(OH)_2D_3$. Therefore, the VDR antagonistic potency of (23*S*,25*S*)-DLAM-2P was investigated using reporter gene assay in MVNP-transduced NIH3T3 cells. 10^{-9}M 1α,25-$(OH)_2D_3$ significantly increased reporter gene activity as reported previously [26]. On the contrary, (23*S*,25*S*)-DLAM-2P never increased it even at 10^{-6}M. However, (23*S*,25*S*)-DLAM-2P dose-dependently (10^{-9}M to 10^{-6}M) inhibited

the reporter gene activity induced by 10^{-9}M 1α,25-$(OH)_2D_3$. (23*S*,25*S*)-DLAM-2P, which about 10 times high concentration of 1α,25-$(OH)_2D_3$ showed 50% inhibition of 1α,25-$(OH)_2D_3$ action (Figure 6).

Discussion

TEI-9647, which is 1α,25-$(OH)_2D_3$-26,23-lactone analogue, functions as a vitamin D antagonist through VDR-VDRE mediated genomic actions of 1α,25-$(OH)_2D_3$ in human cells, but shows vitamin D agonistic actions in rodent cells [14]. Recently, it was reported that (23*S*,25*S*)-DLAM-1P, a 1α,25-$(OH)_2D_3$-26,23-lactone analogue, showed vitamin D antagonistic action on HL-60 cell differentiation induced by 1α,25-$(OH)_2D_3$ [9]. In the present study, we examined whether 1α,25-$(OH)_2D_3$-26,23-lactam analogues show vitamin D antagonistic action by human cells and rodent cells through a VDR-mediated genomic action of 1α,25-$(OH)_2D_3$. TEI-9647 inhibited 25-OH-D_3-24- hydroxylase gene expression induced by 1α,25-$(OH)_2D_3$ in human cells but not rodent cells. On the other hand, (23*S*,25*R*)-diastereoisomers of 1α,25-$(OH)_2D_3$-26,23-lactam analogues blocked the vitamin D-dependent gene expression by not only human cells but also rodent cells treated with 1α,25-$(OH)_2D_3$. These results indicate that at least (23*S*,25*S*)-diastereoisomers of 1α,25-$(OH)_2D_3$-26,23-lactam analogues function as VDR antagonists that block the VDR-mediated genomic action of 1α, 25-$(OH)_2D_3$ in multiple species.

As for OCL formation, we previously reported that the (23*S*,25*R*)- 1α,25-$(OH)_2D_3$-26,23-lactone, a major metabolite of 1α,25-$(OH)_2D_3$ in vitro and in vivo, blocks OCL formation by human and mouse bone marrow cells treated with 1α,25-$(OH)_2D_3$ through a VDR independent mechanism [24,28,29, Figure 5]. (23*S*,25*R*)-DLAM-1P and (23*S*,25*R*)-DLAM-2P, which are analogues of 1α,25-$(OH)_2D_3$-26,23-lactam, even at 10^{-6}M, never or slightly inhibited VDR-mediated gene expression induced by 10^{-8}M 1α,25-$(OH)_2D_3$. However, 10^{-8} M to 10^{-6}M of these compounds dose-dependently blocked OCL formation by human and mouse bone marrow cultures treated with 10^{-8}M 1α,25-$(OH)_2D_3$ (Figures 4 and 5). These results suggest that 1α,25-$(OH)_2D_3$-26,23-lactam analogues have two mechanism of inhibitory actions on OCL formation; one is a VDR-mediated antagonistic action and the other is VDR-independent 1α,25-$(OH)_2D_3$-26,23-lactone-like action. The mechanism of VDR-independent 1α,25-$(OH)_2D_3$-26,23-lactone-like action is still unknown.

The mechanism of VDR antagonistic action of TEI-9647 and 1α,25-$(OH)_2D_3$-26,23-lactam analogues is quite different. Recently, we demonstrated that the C-terminal region of the ligand binding domain of VDR plays an important role in determining the antagonistic/agonistic profile of TEI-9647 and suggested that one or both of the two cysteine residues at 403 and 410 in human VDR are key residues for the antagonistic activity of TEI-9647 [14,15]. Unlike TEI-9647, ZK159222 and ZK168281, which are also VDR antagonists, exhibited their antagonistic effect regardless of the species of VDR. This suggests the molecular mechanisms responsible for the antagonistic effects of TEI-9647, and ZK159222 or ZK168281 are different, although both types of compounds have relatively bulky ring structures in their side chains compared with 1α,25-$(OH)_2D_3$. However, the TEI-9647 side chain is not as bulky as that of ZK159222 and ZK168281. The extended side chains of ZK159222 and ZK168281 prevent the interaction between residues H397 and F422 [16,30,31]. Disturbance of the interaction with these residues makes helix 12 deviate from an optimized position for coactivator recruitment. In the case of 1α,25-$(OH)_2D_3$-26,23-lactam analogues, 1α,25-$(OH)_2D_3$-26,23-lactam, N-methyl- and N-isopropyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues never act as vitamin D antagonists (unpublished data). On the contrary, N-benzyl-, N-phenetyl- and N-phenyl-butyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues are vitamin D antagonists in human and rodent cells [19]. These results demonstrate that compounds which have a bulky group with a nitrogen atom in the lactam ring of 1α,25-$(OH)_2D_3$-26,23-lactam analogues showed VDR antagonistic

action. The molecular mechanism responsible for the vitamin D antagonistic actions of N-benzyl- and N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues may be that they block the conformational change of helix 12 in VDR, and prevent coactivator recruitment, but the exact mechanism of action of these compounds is still unclear.

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Abbreviations

1α
: 25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3

1α
: 25-$(OH)_2D_3$-26,23- lactone, (23*S*,25*R*)-1 α,25-dihydroxyvitamin D_3-26,23-lactone

TEI-9647
: (23*S*)-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactone

TEI-9648
: (23*R*)-25-dehydro-1α-hydroxyvitamin D_3- 26,23-lactone

1α
: 25-$(OH)_2D_3$-26,23-lactam, 1α,25-dihydroxy-vitamin D_3-26,23-lactam

DLAM-1P
: N-Benzyl-1α,25-dihydroxyvitamin D_3-26,23-lactam

DLAM-2P
: N-Phenetyl- 1α,25-dihydroxyvitamin D_3-26,23-lactam

25-OH-D_3-24-hydroxylase
: 25-hydroxyvitamin D_3- 24-hydroxylase

VDR
: vitamin D receptor

VDRE
: vitamin D responsive element

MVNP
: measles virus nucleocapsid protein

CFU-GM
: colony forming unit-granulocyte macrophage

M-CSF
: macrophage-colony stimulating factor

GM-CSF
: granulocyte- macrophage colony stimulating factor

SCF

stem cell factor

IL-3
interleukin-3

IL-6
interleukin-6

OCL
osteoclast

NBT
nitro blue tetrazolium

TRAP
tartrate-resistant acid phosphatase

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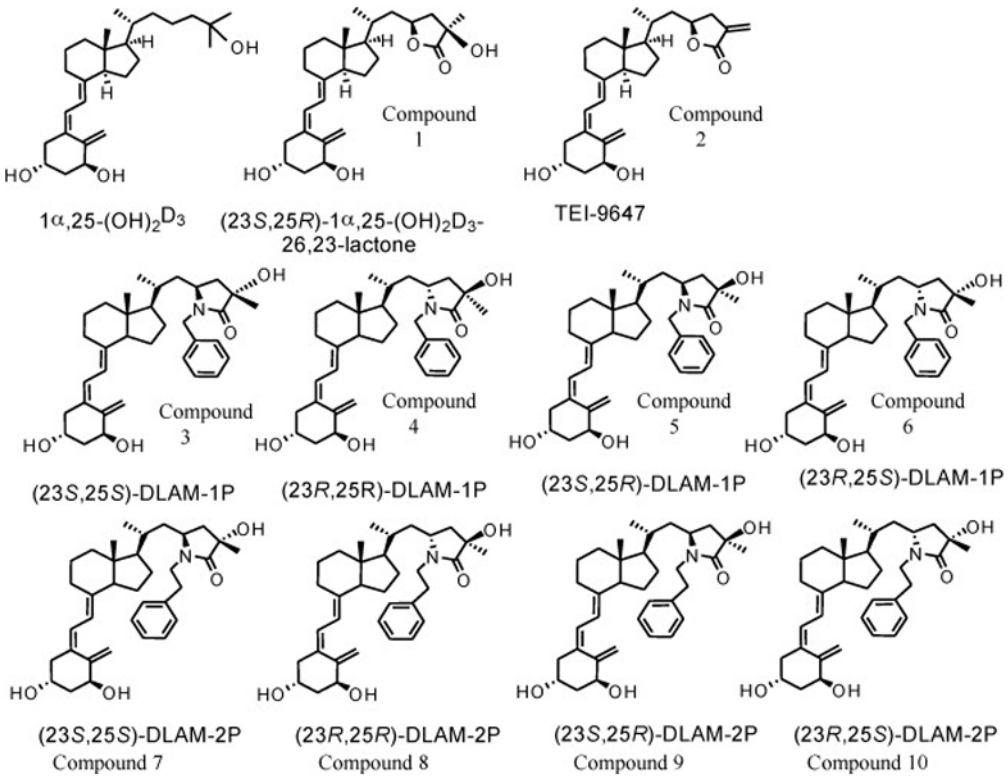

Figure 1.

Structures of 1α,25-$(OH)_2D_3$-26,23-lactone analogues and 1α,25-$(OH)_2D_3$-26,23-lactam analogues. The (23*S*,25*R*)-1α,25-$(OH)_2D_3$-26,23-lactone is a naturally occurring metabolite derived from 1α,25-$(OH)_2D_3$.

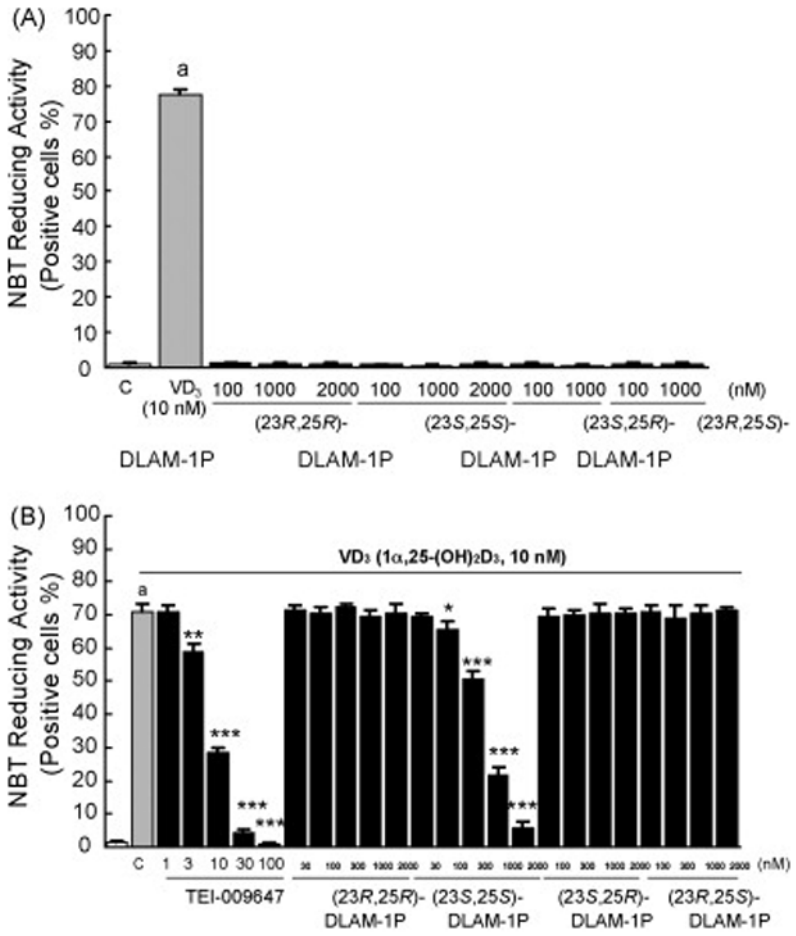

Figure 2.

Effects of 4 diastereoisomers of N-benzyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues on 1α,25-$(OH)_2D_3$-induced HL-60 cell differentiation as determined by NBT-reducing activity. (A) HL-60 cells were treated with 1α,25-$(OH)_2D_3$ alone or N-benzyl-1α,25-$(OH)_2D_3$-26,23-lactam alone for 96 hrs, and NBT-reducing activity was examined. (B) HL-60 cells were treated with N-benzyl-1α,25-$(OH)_2D_3$-26,23-lactam in the presence of 10^{-8}M 1α,25-$(OH)_2D_3$ for 96 hrs, and NBT-reducing activity was examined. Rectangles and bars show mean ± S.D. of triplicate experiments, respectively. [a]$p<0.001$, compared with cells treated with media alone. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, compared with cells treated with 10^{-8}M 1α,25-$(OH)_2D_3$, respectively. Similar results were seen in two independent experiments.

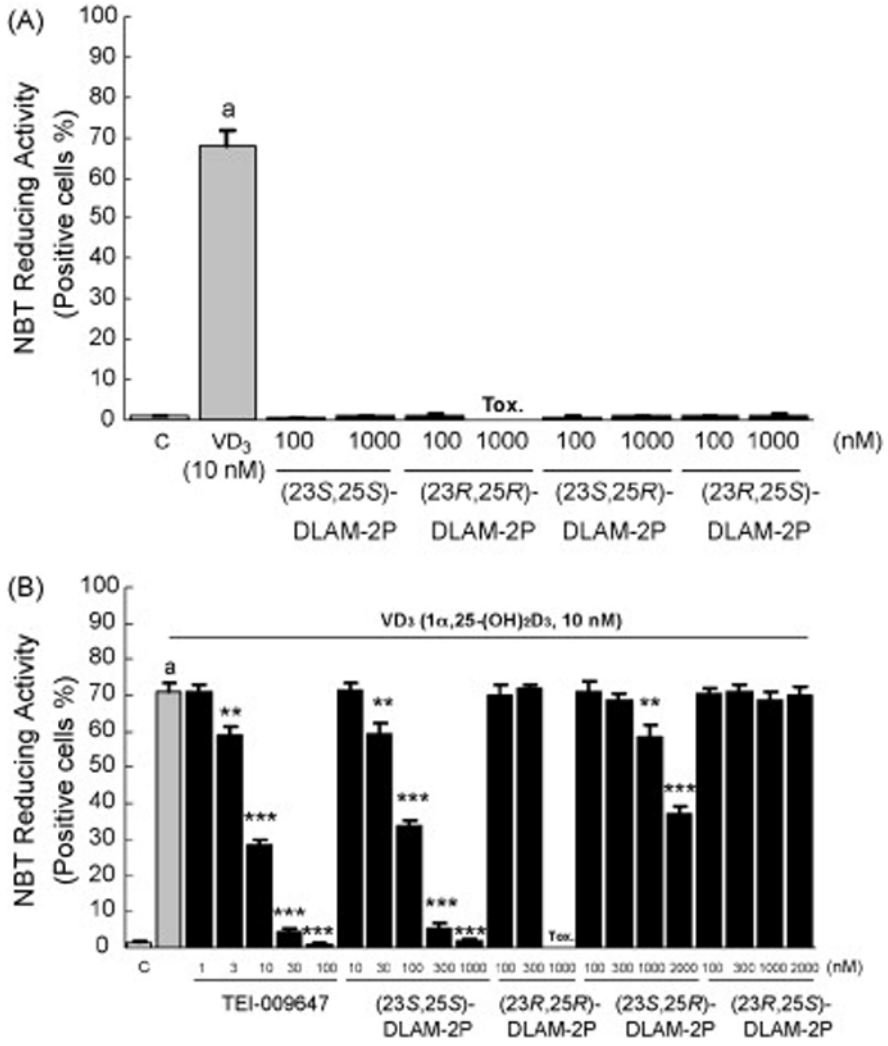

Figure 3.

Effects of 4 diastereoisomers of N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues on 1α, 25-$(OH)_2D_3$-induced HL-60 cell differentiation as determined by NBT-reducing activity. (A) HL-60 cells were treated with 1α,25-$(OH)_2D_3$ alone or N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam alone for 96 hrs, and NBT-reducing activity was examined. (B) HL-60 cells were treated with N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam in the presence of 10^{-8}M 1α,25-$(OH)_2D_3$ for 96 hrs, and NBT-reducing activity was examined. Rectangles and bars show mean ± S.D. of triplicate experiments, respectively. [a]$p<0.001$, compared with cells treated with media alone. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, compared with cells treated with 10^{-8}M 1α,25-$(OH)_2D_3$, respectively. Similar results were seen in two independent experiments.

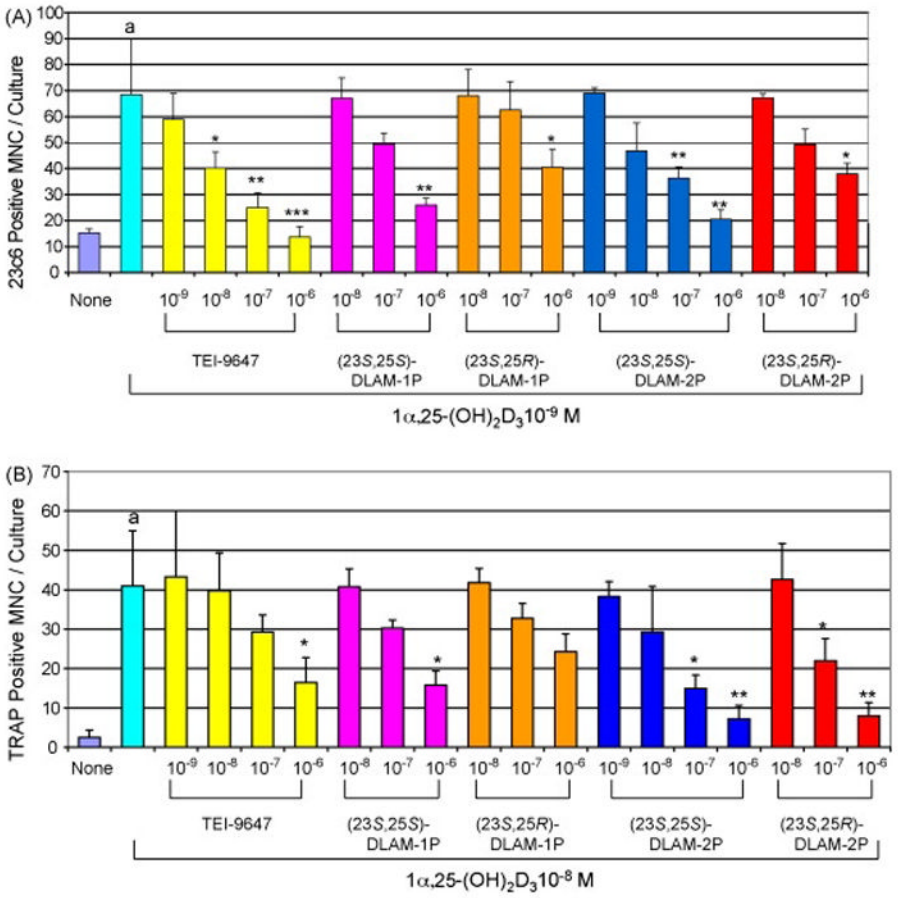

Figure 4.

Effects of N-benzyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues and N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues on OCL formation by human MVNP-transduced CFU-GM cells (A) and mouse bone marrow cells (B) treated with 1α,25-$(OH)_2D_3$. The experimental procedures for OCL formation from human MVNP-transduced CFU-GM cells and mouse bone marrow cells were carried out as described in Materials and Methods. Multinucleated cells that cross-reacted with the 23C6 antibody in human MVNP-transduced CFU-GM cells and TRAP-positive in mouse bone marrow cells and had three or more nuclei were scored an OCL. Data are expressed as the mean ± S.D. of quadruplicate determinations. [a]$p<0.001$, compared with cells treated with media alone. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, compared with cells treated with 10^{-9}M 1α,25-$(OH)_2D_3$ or 10^{-8}M 1α,25-$(OH)_2D_3$, respectively. Similar results were seen in two independent experiments.

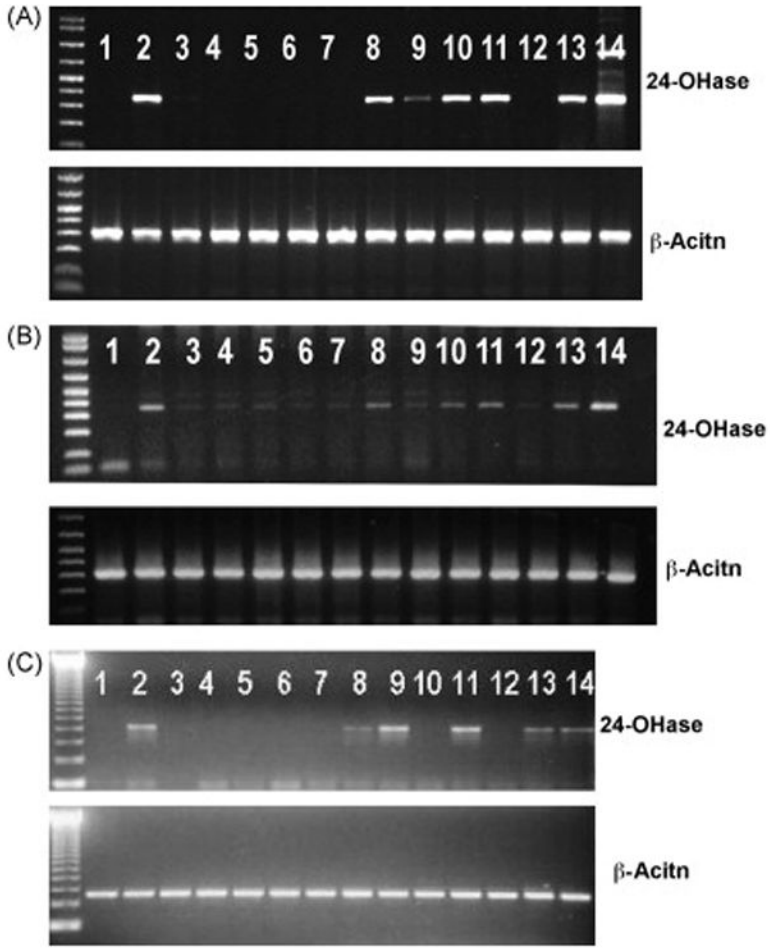

Figure 5.

Effects of N-benzyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues and N-phenetyl-1α,25-$(OH)_2D_3$-26,23-lactam analogues on 25-OH-D_3-24-hydroxylase gene expression induced by 1α,25-$(OH)_2D_3$ in HL-60 cells (A), human osteoblastic cell line (HOS cells) (B) and primary mouse osteoblastic cells (C). The experimental procedures for gene expression induced by vitamin D analogues were carried out as described in Materials and Methods. 1, vehicle; 2, 10^{-8}M 1α,25-$(OH)_2D_3$; 3, 10^{-6}M TEI-9647; 4, 10^{-6}M (23*S*,25*S*)-DLAM-1P; 5, 10^{-6}M (23*S*,25*R*)-DLAM-1P; 6, 10^{-6}M (23*S*,25*S*)-DLAM-2P; 7, 10^{-6}M (23*S*,25*R*)-DLAM-2P; 8, 10^{-6}M 1α,25-$(OH)_2D_3$-26,23-lactone; 9, 10^{-8}M 1α,25-$(OH)_2D_3$ + 10^{-6}M TEI-9647; 10, 10^{-8}M 1α,25-$(OH)_2D_3$ + 10^{-6}M (23*S*,25*S*)-DLAM-1P; 11, 10^{-8}M 1α,25-$(OH)_2D_3$ + 10^{-6}M (23*S*,25*R*)-DLAM-1P; 12, 10^{-8}M 1α,25-$(OH)_2D_3$ + 10^{-6}M (23*S*,25*S*)-DLAM-2P; 13, 10^{-8}M 1α,25-$(OH)_2D_3$ + 10^{-6}M (23*S*,25*R*)-DLAM-2P; 14, 10^{-8}M 1α,25-$(OH)_2D_3$ + 10^{-6}M 1α,25-$(OH)_2D_3$-26,23-lactone. Similar results were seen in two independent experiments.

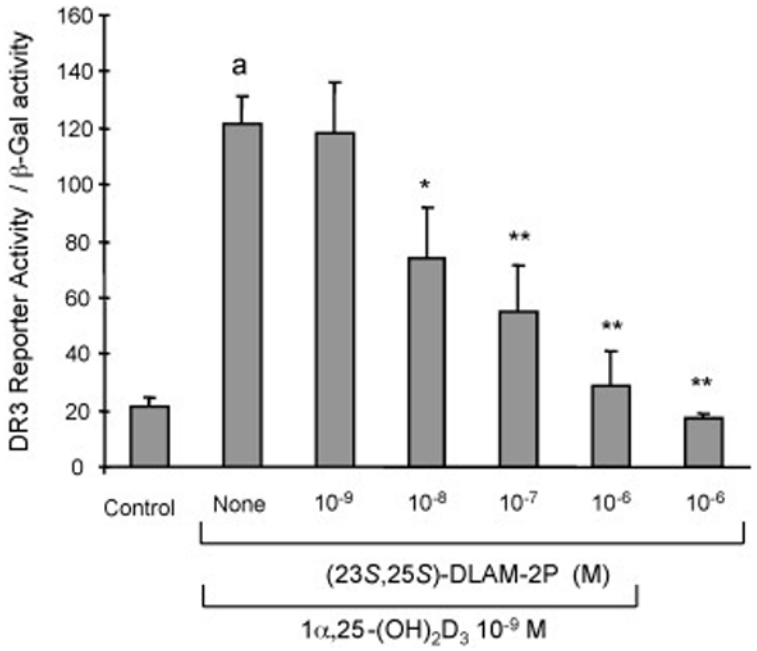

Figure 6.

Effect of (23*S*,25*S*)-DLAM-2P on reporter gene activity induced by 1α,25-$(OH)_2D_3$ in MVNP-transduced NIH3T3 cells. The reporter plasmid containing 25-OH-D_3-24-hydroxylase promoter and β-galactosidase vector were transfected into MVNP-transduced NIH3T3 cells. 10^{-9}M 1α,25-$(OH)_2D_3$ and various concentration of (23*S*,25*S*)-DLAM-2P were added for 24 hours after the transfection of MVNP-transduced NIH3T3 cells. Results are expressed as the mean ± SEM. [a]$p<0.001$, compared with cells treated with media alone. *$p< 0.01$ and **$p<0.001$ compared with cells treated with 10^{-9}M 1α,25-$(OH)_2D_3$. Similar results were seen in three independent experiments.

Table 1

Binding affinities of 1α,25-$(OH)_2D_3$-26,23-lactam analogues to competitively inhibit [^{3}H]1α,25-$(OH)_2D_3$ binding to chick intestinal VDR

Compounds	50% Displacement (pg/tube)	Molar Ratio
1α,25-$(OH)_2D_3$	37.5	1
(23*S*,25*S*)-DLAM-1P	1,750	36.4
(23*R*,25*R*)-DLAM-1P	19,000	395.5
(23*S*,25*R*)-DLAM-1P	28,000	567.9
(23*R*,25*S*)-DLAM-1P	20,000	405.7
(23*S*,25*S*)-DLAM-2P	620	12.6
(23*R*,25*R*)-DLAM-2P	9,700	196.7
(23*S*,25*R*)-DLAM-2P	14,500	294.1
(23*R*,25*S*)-DLAM-2P	25,500	517.2

Molar ratio indicates the ratio of moles per liter of 1α,25-$(OH)_2D_3$ analogues over the moles per liter of 1α,25-$(OH)_2D_3$ required for 50% displacement of the [^{3}H]1α,25-$(OH)_2D_3$ from the receptor. Each value of 50% displacement was calculated as the mean for duplicate determinations.

Table 2

Comparison of the OCL formation inhibitory actions of 1α,25-$(OH)_2D_3$-26,23-lactam analogues between human MVNP-transduced CFU-GM cultures and mouse bone marrow cultures.

Compounds	OCL formation inhibitory action (IC_{50})	
	10^{-9}M 1α,25-$(OH)_2D_3$ in MVNP-transduced CFU-GM cultures	10^{-8}M 1α,25-$(OH)_2D_3$ in mouse bone marrow cultures
TEI-9647	7.5×10^{-9}M	1.5×10^{-7}M
(23*S*,25*S*)-DLAM-1P	2.1×10^{-7}M	1.8×10^{-7}M
(23*S*,25*R*)-DLAM-1P	8.6×10^{-7}M	5.0×10^{-7}M
(23*S*,25*S*)-DLAM-2P	4.0×10^{-8}M	1.5×10^{-8}M
(23*S*,25*R*)-DLAM-2P	3.9×10^{-7}M	4.4×10^{-8}M

The IC_{50} values for the OCL formation inhibitory action of 1α,25-$(OH)_2D_3$- 26,23-lactam were calculated from the results in Figure 4.