1α,25-Dihydroxyvitamin D₃-26,23-lactam, a Novel Vitamin D₃ Analog, Acts as a Vitamin D₃ Antagonist in Human Prostate Cancer Cells

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1α,25-Dihydroxyvitamin D₃ [1α,25(OH)₂D₃], the active form of vitamin D₃, is known to exhibit an anti-tumor properties and markedly suppresses the growth of various human cancer cells. We synthesized novel vitamin D analogs, 1α,25-dihydroxyvitamin D₃-26,23-lactam (DLAMs), having a lactam moiety in the side chain, and examined the effects on cell growth of human prostate cancer cells LNCaP. 1α,25(OH)₂D₃ significantly suppressed both the number of cells and cell viability. The mRNA expression of p21, well-known as a tumor suppressive gene, was clearly induced by treatment with 1α,25(OH)₂D₃ in LNCaP cells. The effects of 1α,25(OH)₂D₃ on the growth suppression of LNCaP cells was attenuated by the simultaneous addition of (23S,25S)-DLAM-1P. In a computer docking simulation, (23S,25S)-DLAM-1P bound to vitamin D receptor (VDR), and its lactam moiety may interfere VDR helix-12 folding. Its stereoisomer (23R,25R)-DLAM-1P did not influence cell growth regulated by 1α,25(OH)₂D₃. The expression of p21 mRNA induced by 1α,25(OH)₂D₃ was suppressed by (23S,25S)-DLAM-1P but not by (23R,25R)-DLAM-1P in LNCaP cells. In the absence of 1α,25(OH)₂D₃, neither (23S,25S)-DLAM-1P nor (23R,25R)-DLAM-1P regulated the cell growth of LNCaP cells. Thus, (23S,25S)-DLAM-1P interferes with the VDR signal and acts as a vitamin D₃ antagonist in cancer cells.

Key words —— vitamin D, cancer, 1α,25-dihydroxyvitamin D₃-26,23-lactam

INTRODUCTION

1α,25-Dihydroxyvitamin D₃ [1α,25(OH)₂D₃] is an active form of vitamin D₃, and exhibits various physiological actions, including the regulation of intestinal calcium absorption and bone metabolism. In addition, previous studies have shown that 1α,25(OH)₂D₃ could suppress the cell growth of cancer cells such as myeloid leukemia, colon cancer and breast cancer cells in vitro and in vivo.1–3 Many epidemiological studies show associations between vitamin D status and the risk and mortality rates of a number of cancers such as colorectal, breast and prostate cancers.4–6 In studies using human cancer cells, 1α,25(OH)₂D₃ exhibits anti-proliferative activity mainly by regulation of the cell cycle of cancer cells. Progression through the cell cycle is regulated by cyclins, and their association with cyclin-dependent kinase (CDK) and CDK inhibitors (CKIs) such as p21 and p27. Expression of CKIs inhibits proliferation by inducing G1 cell cycle arrest and withdrawal from the cell cycle to the G0 phase. Regulation of the CDK-dependent cell cycle may be a main target for the mechanism of anti-proliferative activity of 1α,25(OH)₂D₃ in cancer cells.7

It is known that 1α,25(OH)₂D₃ binds to vitamin D receptor (VDR), a member of the nuclear receptor super-family, and exhibits its biological function in target cells. VDR possesses a DNA-binding domain and a ligand-binding domain (LBD), which is formed by 12 α-helices, containing an activation function 2 (AF-2) domain.8 The carboxyl-terminal α-helix (helix 12) plays an important role in the binding and transcriptional activity of the VDR-1α,25(OH)₂D₃ complex. Numerous vitamin D analogs have been synthesized and reported, but vitamin D antagonists were rare in previous studies. We have recently introduced novel...
reverse transcriptase (RT)-PCR Analysis ——

Total RNA was extracted from cultured LNCaP cells, using the acid guanidium-phenol-chloroform method. cDNA was synthesized from 5μg of total RNA by RT (Superscript II Preamplification System, Invitrogen, Carlsbad, CA, U.S.A.) and amplified using PCR. The primers used in PCR for the human p21 gene were 5′-GAT GTC CGT CAG AAC CCA TG-3′ (sense primer) and 5′-TTA GGG CTT CCA GCC AAC-3′ (anti-sense primer). The reaction conditions for PCR were 26 cycles, denaturation at 94°C for 45 s, annealing at 59°C for 45 s, and extension at 72°C for 2 min. The primers used in PCR for the human VDR gene were 5′-CTA TTC ACC TGC CCC TTC AAC-3′ (sense primer) and 5′-GCT CCC TCC TTC AAC-3′ (anti-sense primer). The reaction conditions for PCR were 27 cycles, denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 2 min. The primers used in PCR for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were 5′-CAT GGA GAA GGC TGG GGC TC-3′ (sense primer) and 5′-AGG CAG GGA TGA TGT TTC AAC-3′ (anti-sense primer). The reaction conditions for PCR were 18 cycles, denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 2 min. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide.

Statistical Analysis —— Data are expressed as the means ± standard deviation. The significance of differences was analyzed using Student’s t test.

RESULTS

Structure of DLAM-1P

We have recently introduced novel 1α,25(OH)_{2}D_{3} analogs, DLAMs. In the present study, we newly synthesized stereoisomers, (23R,25R)-DLAM-1P and (23S,25S)-DLAM-1P, as shown in Fig. 1. We characterized their biological activity.

MATERIALS AND METHODS

Cells and Reagents —— A human prostate cancer cell line, LNCaP, was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). LNCaP cells were cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS) at 37°C under 5% CO_{2} in air. DLAMs, including (23S,25S)-DLAM-1P and (23R,25R)-DLAM-1P, were synthesized in our laboratory as shown in our previous studies.10 1α,25(OH)_{2}D_{3} was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Measurement of Cell Growth —— Cell growth of LNCaP cells was monitored by cell counting in vitro cultures, and cell viability of LNCaP cells was measured by WST-8 assay. For cell counting, LNCaP cells (5×10^{4} cells) were cultured in 0.5 ml of RPMI1640 supplemented with 10% FCS on a 24-well plate. After being cultured for 1–3 days, the cells were detached by 0.25% trypsin/0.02%EDTA/phosphate buffered saline and counted using a hemocytometer. For the WST-8 assay, LNCaP cells (2×10^{4} cells) were cultured in 0.1 ml of RPMI1640 supplemented with 10% FCS on a 96-well plate. After being cultured for 3 days, the cells were assayed using a WST-8 assay kit (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan). Cell viability is expressed as a percentage of the control.

1α,25(OH)_{2}D_{3} analogs, 1α,25-dihydroxyvitamin D_{3}-26,23-lactam (DLAMs), which have a lactam structure in the side chain. We first reported that 1α,25(OH)_{2}D_{3} could induce differentiation into mature macrophages in human myeloid leukemia cells HL-60.11 The assay system has been widely used in the screening of vitamin D analogs to check their biological activity. In the assay, DLAMs inhibit 1α,25(OH)_{2}D_{3}-induced differentiation of HL-60 cells, but the effects of DLAMs on the proliferation of human cancers, including prostate cancers, is not known.

1α,25(OH)_{2}D_{3} regulates cell growth and various gene expression of human prostate cancer, LNCaP cells. In the present study, we examined the effects of two stereoisomers of DLAMs on 1α,25(OH)_{2}D_{3}-dependent regulation of cell growth and gene expression in LNCaP cells in vitro cultures. (23S,25S)-DLAM-1P, a stereoisomer of DLAM, antagonized 1α,25(OH)_{2}D_{3}-induced suppression of cell growth by the regulation of p21 expression. Our data suggest, for the first time, that DLAM is a novel antagonist of 1α,25(OH)_{2}D_{3} in cancer cell growth, and that a lactam structure in the side chain may offer a new approach for the development of vitamin D analogs for the prevention of prostate cancer.
biological function.

Effects of 1α,25(OH)2D3 on Cell Growth and Expression of p21 mRNA in Human Prostate Cancer Cells, LNCaP

We first examined the effects of 1α,25(OH)2D3 on cell growth of human prostate cancer cells, LNCaP. When LNCaP cells were cultured for 3 days, 1–100 nM 1α,25(OH)2D3 suppressed the number of cells on day 2 and 3 in a dose-dependent manner (Fig. 2 A). Cell viability measured by WST-8 assay was also suppressed by adding 1–100 nM 1α,25(OH)2D3 on day 3 in LNCaP cells (Fig. 2 B). To examine the influence of 1α,25(OH)2D3 on the cell cycle of LNCaP cells, the expression of p21 mRNA was measured by RT-PCR. LNCaP cells constitutively expressed p21, CDK inhibitor, and VDR mRNAs, and 1α,25(OH)2D3 induced the expression of p21, but not VDR in LNCaP cells (Fig. 2 C), suggesting that 1α,25(OH)2D3 inhibits the proliferation of LNCaP cells via p21-dependent suppression of G1/S transition of the cell cycle.

Influence of DLAM-1P on Cell Growth of LNCaP Cells

We next examined the effects of (23R,25R)-DLAM-1P and (23S,25S)-DLAM-1P on the growth of LNCaP cells in the presence or absence of 1α,25(OH)2D3. When LNCaP cells were treated with (23R,25R)-DLAM-1P and (23S,25S)-DLAM-1P, both DLAMs did not influence the proliferation of LNCaP cells in the absence of 1α,25(OH)2D3 (Fig. 3 A). With the simultaneous addition of
Fig. 3. Effects of DLAM-1P on Cell Growth of LNCaP Cells in the Presence or Absence of 1α,25(OH)2D3 (A) LNCaP cells (5 × 10^4 cells) were cultured for 3 days with or without 10 nM 1α,25(OH)2D3, 1 µM (23R,25R)-DLAM-1P, and 1 µM (23S,25S)-DLAM-1P. Cell growth was determined by cell counting.
(B) LNCaP cells (5 × 10^4 cells) were cultured for 3 days with or without 1 µM (23R,25R)-DLAM-1P and 1 µM (23S,25S)-DLAM-1P in the presence of 10 nM 1α,25(OH)2D3. Cell growth was determined by counting. Significantly different, *p < 0.01, **p < 0.001 versus control, #p < 0.01 versus 1α,25(OH)2D3. Data are expressed as the means ± SEM of 3 wells.

1α,25(OH)2D3 and DLAM, the suppression of cell growth induced by 1α,25(OH)2D3 was abolished by (23S,25S)-DLAM-1P (Fig. 3 B). Its stereoisomer (23R,25R)-DLAM-1P did not influence cell growth regulated by 1α,25(OH)2D3.

Influence of DLAM-1P on the Expression of p21 and VDR mRNAs in LNCaP Cells

To clarify the action mechanism of DLAM in the growth of cancer cells, we examined the expression of p21 and VDR mRNAs in LNCaP cells in the presence of 1α,25(OH)2D3. Total RNA was extracted from LNCaP cells 9 hr after the addition of these vitamin D analogs. When LNCaP cells were treated with both (23S,25S)-DLAM-1P and 1α,25(OH)2D3, the expression level of p21 mRNA induced by 10 nM 1α,25(OH)2D3 was suppressed by 1 µM (23S,25S)-DLAM-1P (Fig. 4). (23R,25R)-DLAM-1P did not influence the expression of p21 mRNA regulated by 1α,25(OH)2D3. The level of VDR expression in LNCaP cells was not influenced by 1α,25(OH)2D3 and DLAMs.

DISCUSSION

In human prostate cancer cells, LNCaP, 1α,25(OH)2D3 significantly suppressed cell growth by cell-cycle arrest by inducing p21 expression. The anti-tumor effects of 1α,25(OH)2D3 in LNCaP cells were attenuated by the simultaneous addition of (23S,25S)-DLAM-1P. Its stereoisomer, (23R,25R)-DLAM-1P, did not influence cell growth regulated by 1α,25(OH)2D3. Neither (23S,25S)-DLAM-1P nor (23R,25R)-DLAM-1P regulated the growth of LNCaP cells in the absence of 1α,25(OH)2D3.

1α,25(OH)2D3 is known to bind to VDR in target cells to exhibit its biological and pharmacological functions, including intestinal calcium absorption, bone metabolism, immune regulation and anti-tumor activity. In our previous study, DLAMs showed affinity for VDR, and DLAM derivatives with (23S,25S) stereochemistries showed higher VDR affinity than other corresponding (23R,25R) diastereoisomers in a competitive receptor binding assay using chick intestinal VDR.
was about 1/37 of that of 1α,25(OH)2D3.10) On the other hand, the VDR binding affinities of (23R,25R)-DLAM-1P were very weak, less than 1/200, compared with 1α,25(OH)2D3.10) In the present study, 100-fold concentration (1 μM) of (23S,25S)-DLAM-1P was needed to attenuate the anti-tumor activity induced by 10 nM 1α,25(OH)2D3 (Figs. 3 and 4). DLAMs possess a benzyl group on the nitrogen atom in the lactam structure in the side chain (Fig. 1), and this bulky structure may influence binding to VDR-LBD and the biological function. In a computer docking simulation, the bulky benzyl group in the lactam moiety of (23S,25S)-DLAM-1P interfered with the Phe422 residue in helix 12 of VDR-LBD (data not shown), therefore, (23S,25S)-DLAM-1P may bind to VDR in LNCaP cells and act as an antagonist for 1α,25(OH)2D3 due to interference by VDR-LBD.

Epidemiological studies have shown the important concept that exposure to sunlight, which leads to vitamin D synthesis, can reduce the risk of colorectal cancer.15) In addition, there are several observations of an association between low serum 25(OH)D3 levels and increased risk for colorectal, breast and prostate cancers. In animal models of the prostate, ovary, breast and lung cancers, administration of 1α,25(OH)2D3 has significant anticancer effects.16–18) Cell-cycle perturbation is thought to be central mechanism for 1α,25(OH)2D3-mediated anti-proliferative activity in cancer cells. Progression through the cell cycle is regulated by cyclins, and CDK and CKIs, including p21, which are major regulators of cyclin function.19) 1α,25(OH)2D3 could induce the expression of CKIs to inhibit the proliferation of cancer cells by inducing G1 arrest. In the present study, 1α,25(OH)2D3 showed anti-proliferative effects on LNCaP cells by the increased expression of p21, and the effects of 1α,25(OH)2D3 were attenuated by adding (23S,25S)-DLAM-1P, therefore, CKIs are thought to be the major target molecules regulated by 1α,25(OH)2D3 and DLAM-1P in LNCaP cells.

A number of vitamin D derivatives have been synthesized, and some derivatives exhibited vitamin D-like biological activity, but candidate antagonists are rare. 2MD, 2-methylene-19-nor-(20S)-1α,25-(OH)2D3, is reported to act as an agonist in osteoblasts in bone tissues, and its potency was greater than that of 1α,25(OH)2D3.19) ED71, 2β-(3-hydroxypropoxy)-1α,25-(OH)2D3, shows agonistic effects to increase bone mass with less hypercalcemic action than 1α(OH)D3.20) TEI-9647, (23S)-25-dehydro-1α-(OH)D3-26,23-lactone is reported to act as an antagonist of 1α,25(OH)2D3-mediated osteoclast differentiation.21) In the present study, we reported for the first time that (23S,25S)-DLAM-1P interferes with the VDR signal and acts as an antagonist in cancer cells. Vitamin D antagonists have no effects on hypercalcemic action, so it is possible that DLAM derivatives show anti-tumor activity with fewer side effects in the strategy of anticancer therapeutics, and are under investigation in our laboratories.

REFERENCES


