# 1$\alpha$,25-Dihydroxyvitamin $D_3$-26,23-lactam, a Novel Vitamin $D_3$ Analog, Acts as a Vitamin $D_3$ Antagonist in Human Prostate Cancer Cells

**Morichika Takita, Michiko Hirata, Kazuki Tsukamoto, Kazuo Nagasawa, Chisato Miyaura,* and Masaki Inada**

*Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2–24–16 Nakamachi, Koganei, Tokyo 184–8588, Japan*

(Received May 3, 2008; Accepted May 24, 2008)

**1$\alpha$,25-Dihydroxyvitamin $D_3$ [1$\alpha$,25$(OH)_2D_3$], the active form of vitamin $D_3$, is known to exhibit an anti-tumor properties and markedly suppresses the growth of various human cancer cells. We synthesized novel vitamin D analogs, 1$\alpha$,25-dihydroxyvitamin $D_3$-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. 1$\alpha$,25$(OH)_2D_3$ 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 1$\alpha$,25$(OH)_2D_3$ in LNCaP cells. The effects of 1$\alpha$,25$(OH)_2D_3$ on the growth suppression of LNCaP cells was attenuated by the simultaneous addition of (23*S*,25*S*)-DLAM-1P. In a computer docking simulation, (23*S*,25*S*)-DLAM-1P bound to vitamin D receptor (VDR), and its lactam moiety may interfere VDR helix-12 folding. Its stereoisomer (23*R*,25*R*)-DLAM-1P did not influence cell growth regulated by 1$\alpha$,25$(OH)_2D_3$. The expression of p21 mRNA induced by 1$\alpha$,25$(OH)_2D_3$ was suppressed by (23*S*,25*S*)-DLAM-1P but not by (23*R*,25*R*)-DLAM-1P in LNCaP cells. In the absence of 1$\alpha$,25$(OH)_2D_3$, neither (23*S*,25*S*)-DLAM-1P nor (23*R*,25*R*)-DLAM-1P regulated the cell growth of LNCaP cells. Thus, (23*S*,25*S*)-DLAM-1P interferes with the VDR signal and acts as a vitamin $D_3$ antagonist in cancer cells.**

**Key words** —— vitamin D, cancer, 1$\alpha$,25-dihydroxyvitamin $D_3$-26,23-lactam

*To whom correspondence should be addressed: Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2–24–16 Nakamachi, Koganei, Tokyo 184–8588, Japan. Tel.:+81-42-388-7390; Fax: +81-42-388-7390; E-mail: miyaura@cc.tuat.ac.jp

## INTRODUCTION

1$\alpha$,25-Dihydroxyvitamin $D_3$ [1$\alpha$,25$(OH)_2D_3$] is an active form of vitamin $D_3$, and exhibits various physiological actions, including the regulation of intestinal calcium absorption and bone metabolism. In addition, previous studies have shown that 1$\alpha$,25$(OH)_2D_3$ 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, 1$\alpha$,25$(OH)_2D_3$ 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 1$\alpha$,25$(OH)_2D_3$ in cancer cells.[7]

It is known that 1$\alpha$,25$(OH)_2D_3$ 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 $\alpha$-helices, containing an activation function 2 (AF-2) domain.[8] The carboxyl-terminal $\alpha$-helix (helix 12) plays an important role in the binding and transcriptional activity of the VDR-1$\alpha$,25$(OH)_2D_3$ complex. Numerous vitamin D analogs have been synthesized and reported, but vitamin D antagonists were rare in previous studies. We have recently introduced novel

$1\alpha,25(OH)_2D_3$ analogs, $1\alpha$,25-dihydroxyvitamin $D_3$-26,23-lactam (DLAMs), which have a lactam structure in the side chain.[9,10] We first reported that $1\alpha,25(OH)_2D_3$ 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.[12] In the assay, DLAMs inhibit $1\alpha,25(OH)_2D_3$-induced differentiation of HL-60 cells, but the effects of DLAMs on the proliferation of human cancers, including prostate cancers, is not known.

$1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$-dependent regulation of cell growth and gene expression in LNCaP cells *in vitro* cultures. (23*S*,25*S*)-DLAM-1P, a stereoisomer of DLAM, antagonized $1\alpha,25(OH)_2D_3$-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 $1\alpha,25(OH)_2D_3$ 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.

## 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 (23*R*,25*R*)-DLAM-1P and (23*S*,25*S*)-DLAM-1P, were synthesized in our laboratory as shown in our previous studies.[9,10] $1\alpha,25(OH)_2D_3$ 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 ($5\times10^4$ 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 hematocytometer. For the WST-8 assay, LNCaP cells ($2\times10^4$ cells) were cultured in 0.1 ml of RPMI1640 supplemented with 10% FCS on a 96-well plate.[13] 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.

**Reverse transcriptase (RT)-PCR Analysis** —— Total RNA was extracted from cultured LNCaP cells, using the acid guanidium-phenol-chloroform method. cDNA was synthesized from 5 μg 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 CCT CTT GGA GA-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 ACC ATC ATT CAC-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 TCT GG-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 ± scanning electron microscope (SEM). The significance of differences was analyzed using Student's *t* test.

## RESULTS

### Structure of DLAM-1P

We have recently introduced novel $1\alpha,25(OH)_2D_3$ analogs, DLAMs. In the present study, we newly synthesized stereoisomers, (23*R*,25*R*)-DLAM-1P and (23*S*,25*S*)-DLAM-1P, as shown in Fig. 1.[9,10] These DLAMs possess a benzyl group on the nitrogen atom in the lactam structure in the side chain, and this bulky structure may influence binding to VDR-LBD and the

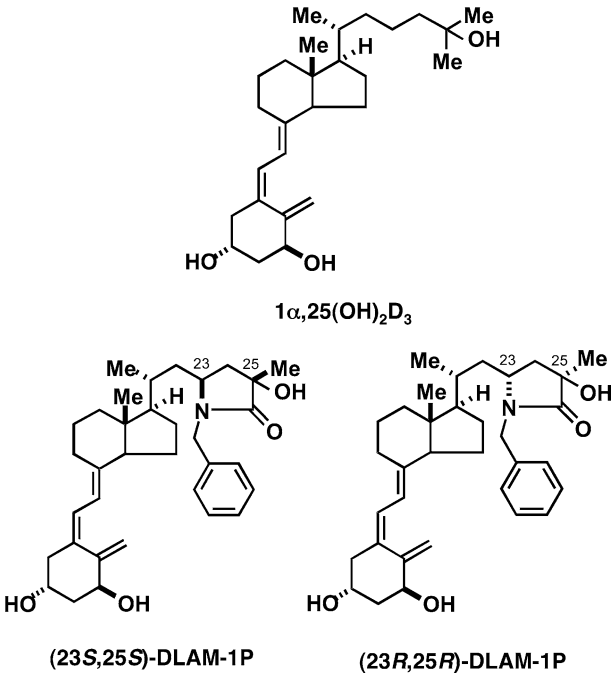


**Fig. 1.** Structures of Active Vitamin $D_3$, $1\alpha,25(OH)_2D_3$, and Newly Synthesized Analogs, DLAMs

biological function.

### Effects of $1\alpha,25(OH)_2D_3$ on Cell Growth and Expression of p21 mRNA in Human Prostate Cancer Cells, LNCaP

We first examined the effects of $1\alpha,25(OH)_2D_3$ on cell growth of human prostate cancer cells, LNCaP. When LNCaP cells were cultured for 3 days, 1–100 nM $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ on day 3 in LNCaP cells (Fig. 2 B). To examine the influence of $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ induced the expression of p21, but not VDR in LNCaP cells (Fig. 2 C), suggesting that $1\alpha,25(OH)_2D_3$ inhibits the proliferation of LNCaP cells via p21-dependent suppression of G1/S transition of the cell cycle.

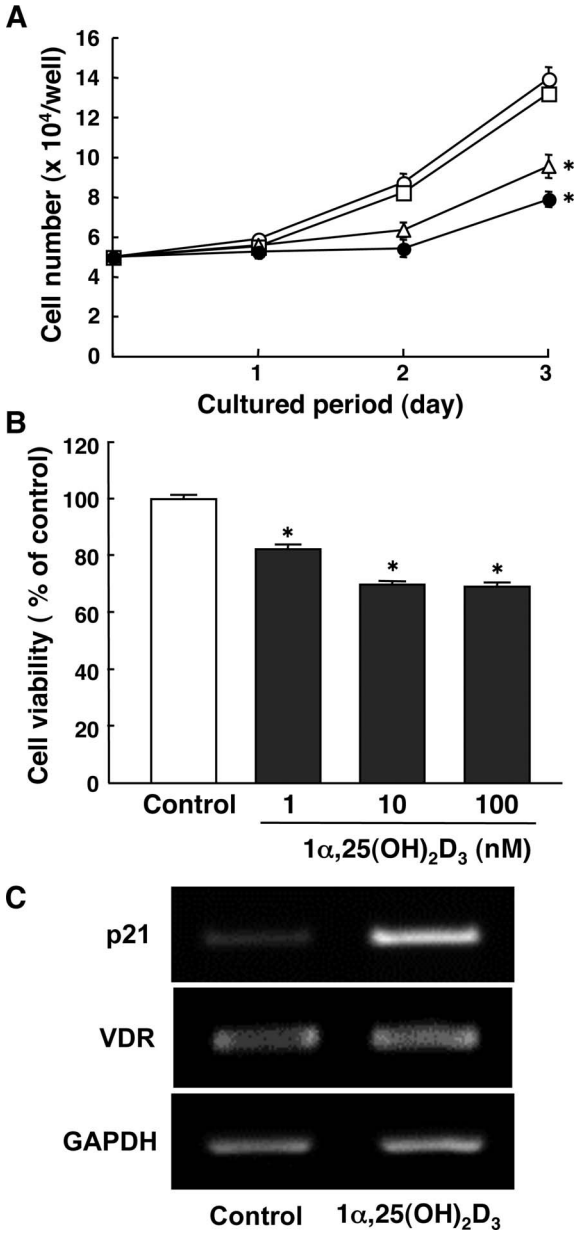


**Fig. 2.** Effects of $1\alpha,25(OH)_2D_3$ on Cell Growth and mRNA Expression of p21 and VDR in Human Prostate Cancer LNCaP Cells

(A) LNCaP cells ($5 \times 10^4$ cells) were cultured for 1–3 days with 0 (○), 1 nM (□), 10 nM (△), or 100 nM (●) $1\alpha,25(OH)_2D_3$. The number of cells was determined by counting. (B) LNCaP cells ($2 \times 10^4$ cells) were cultured for 3 days with or without 1–100 nM $1\alpha,25(OH)_2D_3$. Cell viability was then assayed using a WST-8 assay kit, and expressed as a percentage of the control. Significantly different from the control without $1\alpha,25(OH)_2D_3$, $^*p < 0.001$. Data are expressed as the means ± SEM of 6 wells. (C) LNCaP cells ($5 \times 10^5$ cells) were cultured for 9 hr with or without 100 nM $1\alpha,25(OH)_2D_3$, and then total RNA was extracted. RT-PCR was performed using respective primers for human p21, VDR, and GAPDH.

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

We next examined the effects of (23*R*,25*R*)-DLAM-1P and (23*S*,25*S*)-DLAM-1P on the growth of LNCaP cells in the presence or absence of $1\alpha,25(OH)_2D_3$. When LNCaP cells were treated with (23*R*,25*R*)-DLAM-1P and (23*S*,25*S*)-DLAM-1P, both DLAMs did not influence the proliferation of LNCaP cells in the absence of $1\alpha,25(OH)_2D_3$ (Fig. 3 A). With the simultaneous addition of

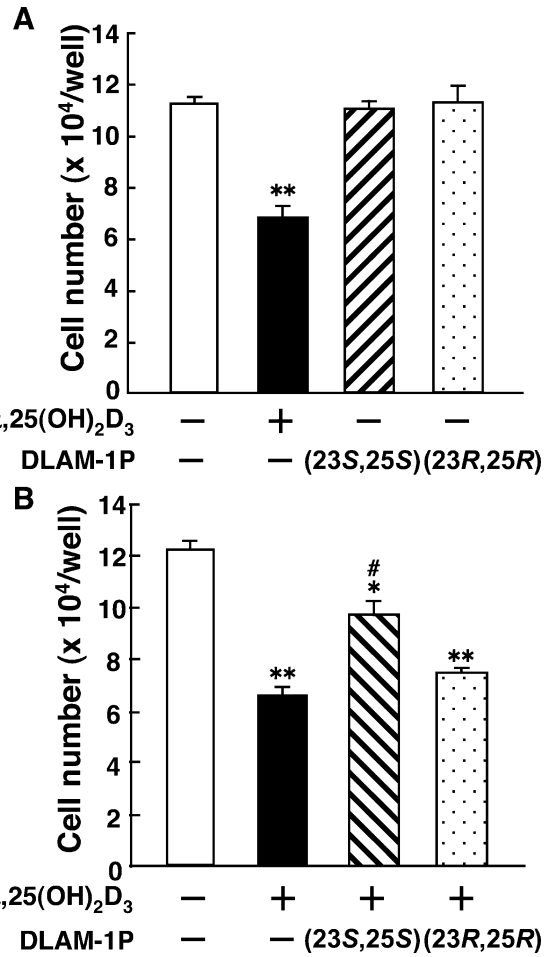


**Fig. 3.** Effects of DLAM-1P on Cell Growth of LNCaP Cells in the Presence or Absence of $1\alpha$,25$(OH)_2D_3$

(A) LNCaP cells ($5 \times 10^4$ cells) were cultured for 3 days with or without 10 nM $1\alpha$,25$(OH)_2D_3$, 1 μM (23*R*,25*R*)-DLAM-1P, and 1 μM (23*S*,25*S*)-DLAM-1P. Cell growth was determined by cell counting. (B) LNCaP cells ($5 \times 10^4$ cells) were cultured for 3 days with or without 1 μM (23*R*,25*R*)-DLAM-1P and 1 μM (23*S*,25*S*)-DLAM-1P in the presence of 10 nM $1\alpha$,25$(OH)_2D_3$. Cell growth was determined by counting. Significantly different, $^*p < 0.01$, $^{**}p < 0.001$ versus control, $^{\#}p < 0.01$ versus $1\alpha$,25$(OH)_2D_3$. Data are expressed as the means ± SEM of 3 wells.

$1\alpha$,25$(OH)_2D_3$ and DLAM, the suppression of cell growth induced by $1\alpha$,25$(OH)_2D_3$ was abolished by (23*S*,25*S*)-DLAM-1P (Fig. 3 B). Its stereoisomer (23*R*,25*R*)-DLAM-1P did not influence cell growth regulated by $1\alpha$,25$(OH)_2D_3$.

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

To clarify the action mechanism of DLAM in the growth of cancer cells, we examined the expression of p21 and VDR mRNA in LNCaP cells in the presence of $1\alpha$,25$(OH)_2D_3$. Total RNA was extracted from LNCaP cells 9 hr after the addition of these vitamin D analogs. When LNCaP cells

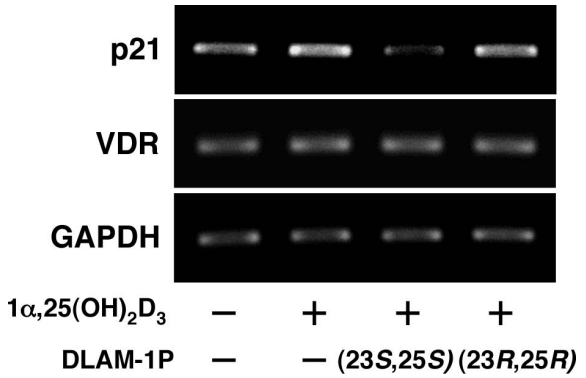


**Fig. 4.** Effects of DLAM-1P on the Expression of p21 and VDR mRNAs in LNCaP Cells

LNCaP cells ($1 \times 10^6$ cells) were cultured for 9 hr in the presence or absence of 10 nM $1\alpha$,25$(OH)_2D_3$, 1 μM (23*R*,25*R*)-DLAM-1P and 1 μM (23*S*,25*S*)-DLAM-1P, and then total RNA was extracted. RT-PCR was performed using respective primers for human p21, VDR, and GAPDH.

were treated with both (23*S*,25*S*)-DLAM-1P and $1\alpha$,25$(OH)_2D_3$, the expression level of p21 mRNA induced by 10 nM $1\alpha$,25$(OH)_2D_3$ was suppressed by 1 μM (23*S*,25*S*)-DLAM-1P (Fig. 4). (23*R*,25*R*)-DLAM-1P did not influence the expression of p21 mRNA regulated by $1\alpha$,25$(OH)_2D_3$. The level of VDR expression in LNCaP cells was not influenced by $1\alpha$,25$(OH)_2D_3$ and DLAMs.

## DISCUSSION

In human prostate cancer cells, LNCaP, $1\alpha$,25$(OH)_2D_3$ significantly suppressed cell growth by cell-cycle arrest by inducing p21 expression. The anti-tumor effects of $1\alpha$,25$(OH)_2D_3$ in LNCaP cells were attenuated by the simultaneous addition of (23*S*,25*S*)-DLAM-1P. Its stereoisomer, (23*R*,25*R*)-DLAM-1P, did not influence cell growth regulated by $1\alpha$,25$(OH)_2D_3$. Neither (23*S*,25*S*)-DLAM-1P nor (23*R*,25*R*)-DLAM-1P regulated the growth of LNCaP cells in the absence of $1\alpha$,25$(OH)_2D_3$.

$1\alpha$,25$(OH)_2D_3$ 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.[14] In our previous study, DLAMs showed affinity for VDR, and DLAM derivatives with (23*S*,25*S*) stereochemistries showed higher VDR affinity than other corresponding (23*R*,25*R*) diastereoisomers in a competitive receptor binding assay using chick intestinal VDR.[10] (23*S*,25*S*)-DLAM-1P exhibited VDR binding affinity, which

was about 1/37 of that of $1\alpha,25(OH)_2D_3$.[10] On the other hand, the VDR binding affinities of (23*R*,25*R*)-DLAM-1P were very weak, less than 1/200, compared with $1\alpha,25(OH)_2D_3$.[10] In the present study, 100-fold concentration (1 μM) of (23*S*,25*S*)-DLAM-1P was needed to attenuate the anti-tumor activity induced by 10 nM $1\alpha,25(OH)_2D_3$ (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 (23*S*,25*S*)-DLAM-1P interfered with the Phe422 residue in helix 12 of VDR-LBD (data not shown), therefore, (23*S*,25*S*)-DLAM-1P may bind to VDR in LNCaP cells and act as an antagonist for $1\alpha,25(OH)_2D_3$ 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 $25(OH)D_3$ levels and increased risk for colorectal, breast and prostate cancers. In animal models of the prostate, ovary, breast and lung cancers, administration of $1\alpha,25(OH)_2D_3$ has significant anticancer effects.[16–18] Cell-cycle perturbation is thought to be central mechanism for $1\alpha,25(OH)_2D_3$-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.[7] $1\alpha,25(OH)_2D_3$ could induce the expression of CKIs to inhibit the proliferation of cancer cells by inducing G1 arrest. In the present study, $1\alpha,25(OH)_2D_3$ showed anti-proliferative effects on LNCaP cells by the increased expression of p21, and the effects of $1\alpha,25(OH)_2D_3$ were attenuated by adding (23*S*,25*S*)-DLAM-1P, therefore, CKIs are thought to be the major target molecules regulated by $1\alpha,25(OH)_2D_3$ 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-(20*S*)-$1\alpha,25-(OH)_2D_3$, is reported to act as an agonist in osteoblasts in bone tissues, and its potency was greater than that of $1\alpha,25(OH)_2D_3$.[19] ED71, $2\beta$-(3-hydroxypropoxy)-$1\alpha,25-(OH)_2D_3$, shows agonistic effects to increase bone mass with less hypercalcemic action than $1\alpha(OH)D_3$.[20] TEI-9647, (23*S*)-25-dehydro-$1\alpha$-$(OH)D_3$-26,23-lactone is reported to act as an antagonist of $1\alpha,25(OH)_2D_3$-mediated osteoclast differentiation.[21] In the present study, we reported for the first time that (23*S*,25*S*)-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


1) Abe, E., Miyaura, C., Sakagami, H., Takeda, M., Konno, K., Yamazaki, T., Yoshiki, S. and Suda, T. (1981) Differentiation of mouse myeloid leukemia cells induced by $1\alpha$,25-dihydroxyvitamin $D_3$. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 4990–4994.
2) Larriba, M. J., Valle, N., Pálmer, H. G., Ordóñez-Morán, P., Alvarez-Díaz, S., Becker, K. F., Gamallo, C., Herreros, A. G., González-Sancho, J. M. and Muñoz, A. (2007) The inhibition of Wnt/$\beta$-catenin signalling by $1\alpha$,25-dihydroxyvitamin $D_3$ is abrogated by Snail1 in human colon cancer cells. *Endocr. Relat. Cancer*, **14**, 141–151.
3) Welsh, J. (2007) Vitamin D and prevention of breast cancer. *Acta Pharmacol. Sin.*, **28**, 1373–1382.
4) Garland, C. F., Comstock, G. W., Garland, F. C., Helsing, K. J., Shaw, E. K. and Gorham, E. D. (1989) Serum 25-hydroxyvitamin D and colon cancer: eight–year prospective study. *Lancet*, **2**, 1176–1178.
5) Bertone-Johnson, E. R., Chen, W. Y., Holick, M. F., Hollis, B. W., Colditz, G. A., Willett, W. C. and Hankinson, S. E. (2005) Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. *Cancer Epidemiol. Biomarkers Prev.*, **14**, 1991–1997.
6) Giovannucci, E., Liu, Y., Rimm, E. B., Hollis, B. W., Fuchs, C. S., Stampfer, M. J. and Willett, W. C. (2006) Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. *J. Natl. Cancer Inst.*, **98**, 451–459.
7) Jensen, S. S., Madsen, M. W., Lukas, J., Binderup, L. and Bartek, J. (2001) Inhibitory effects of $1\alpha$,25-dihydroxyvitamin $D_3$ on the $G_1$-S phase-controlling machinery. *Mol. Endocrinol.*, **15**, 1370–1380.
8) Rochel, N., Wurtz, J. M., Mitschler, A., Klaholz, B. and Moras, D. (2000) The crystal structure of the

nuclear receptor for vitamin D bound to its natural ligand. *Mol. Cell*, **5**, 173–179.

9) Kato, Y., Nakano, Y., Sano, H., Tanatani, A., Kobayashi, H., Shimazawa, R., Koshino, H., Hashimoto, Y. and Nagasawa, K. (2004) Synthesis of 1$\alpha$,25-dihydroxyvitamin $D_3$-26,23-lactams (DLAMs), a novel series of 1$\alpha$,25-dihydroxyvitamin $D_3$ antagonist. *Bioorg. Med. Chem. Lett.*, **14**, 2579–2583.
10) Nakano, Y., Kato, Y., Imai, K., Ochiai, E., Namekawa, J., Ishizuka, S., Takenouchi, K., Tanatani, A., Hashimoto, Y. and Nagasawa, K. (2006) Practical synthesis and evaluation of the biological activities of 1$\alpha$,25-dihydroxyvitamin $D_3$ antagonists, 1$\alpha$,25-dihydroxyvitamin $D_3$-26,23-lactams. Designed on the basis of the helix 12-folding inhibition hypothesis. *J. Med. Chem.*, **49**, 2398–2406.
11) Miyaura, C., Abe, E., Kuribayashi, T., Tanaka, H., Konno, K., Nishii, Y. and Suda, T. (1981) 1$\alpha$,25-Dihydroxyvitamin $D_3$ induces differentiation of human myeloid leukemia cells. *Biochem. Biophys. Res. Commun.*, **102**, 937–943.
12) Shiina, Y., Abe, E., Miyaura, C., Tanaka, H., Yamada, S., Ohmori, M., Nakayama, K., Takayama, H., Matsunaga, I., Nishii, Y., DeLuca, H. F. and Suda, T. (1983) Biological activity of 24,24-difluoro-1$\alpha$,25-dihydroxyvitamin $D_3$ and 1$\alpha$,25-dihydroxyvitamin $D_3$-26,23-lactone in inducing differentiation of human myeloid leukemia cells. *Arch. Biochem. Biophys.*, **220**, 90–94.
13) Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y. and Ueno, K. (1996) A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol. Pharm. Bull.*, **19**, 1518–1520.
14) Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M. T. and Martin, T. J. (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr. Rev.*, **20**, 345–357.
15) Garland, C. F. and Garland, F. C. (1980) Do sunlight and vitamin D reduce the likelihood of colon cancer? *Int. J. Epidemiol.*, **9**, 227–231.
16) Banach-Petrosky, W., Ouyang, X., Gao, H., Nader, K., Ji, Y., Suh, N., DiPaola, R. S. and Abate-Shen, C. (2006) Vitamin D inhibits the formation of prostatic intraepithelial neoplasia in Nkx3.1;Pten mutant mice. *Clin. Cancer Res.*, **12**, 5895–5901.
17) Zhang, X., Jiang, F., Li, P., Li, C., Ma, Q., Nicosia, S. V. and Bai, W. (2005) Growth suppression of ovarian cancer xenografts in nude mice by vitamin D analogue EB1089. *Clin. Cancer Res.*, **11**, 323–328.
18) Nakagawa, K., Kawaura, A., Kato, S., Takeda, E. and Okano, T. (2005) 1$\alpha$,25-Dihydroxyvitamin $D_3$ is a preventive factor in the metastasis of lung cancer. *Carcinogenesis*, **26**, 429–440.
19) Shevde, N. K., Plum, L. A., Clagett-Dame, M., Yamamoto, H., Pike, J. W. and DeLuca, H. F. (2002) A potent analog of 1$\alpha$,25-dihydroxyvitamin $D_3$ selectively induces bone formation. *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 13487–13491.
20) Uchiyama, Y., Higuchi, Y., Takeda, S., Masaki, T., Shiraishi, A., Sato, K., Kubodera, N., Ikeda, K. and Ogata, E. (2002) ED-71, a vitamin D analog, is a more potent inhibitor of bone resorption than alfacalcidol in an estrogen-deficient rat model of osteoporosis. *Bone*, **30**, 582–588.
21) Ishizuka, S., Kurihara, N., Miura, D., Takenouchi, K., Cornish, J., Cundy, T., Reddy, S. V. and Roodman, G. D. (2004) Vitamin D antagonist, TEI-9647, inhibits osteoclast formation induced by 1$\alpha$,25-dihydroxyvitamin $D_3$ from pagetic bone marrow cells. *J. Steroid Biochem. Mol. Biol.*, **89–90**, 331–334.