Mechanism of the anti-proliferative action of 25-hydroxy-19-nor-vitamin D₃ in human prostate cells

Eiji Munetsuna, Sachie Nakabayashi, Rie Kawanami, Kaori Yasuda, Miho Ohta¹, Midori A Arai², Atsushi Kittaka³, Tai C Chen⁴, Masaki Kamakura, Shinichi Ikushiro and Toshiyuki Sakaki

Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

¹Development Nourishment Department, Soai University, Suminoe, Osaka, Japan

²Graduate School of Pharmaceutical Sciences, Chiba University, Inage, Chiba, Japan

³Faculty of Pharmaceutical Sciences, Teikyo University, Sagami, Japan

⁴Boston University School of Medicine, Boston, Massachusetts, USA

(Correspondence should be addressed to T Sakaki; Email: tsakaki@pu-toyama.ac.jp)

Abstract

According to the prevailing paradigm, 1 α -hydroxylation of 25-hydroxyvitamin D₃ (25(OH)D₃) and its analogs is a prerequisite step for their biological effects. We previously reported that 25-hydroxy-19-nor-vitamin D₃ (25(OH)-19-nor-D₃) had anti-proliferative activity in a cell line, PZ-HPV-7, which was derived from human non-cancerous prostate tissue, and suggested that 25(OH)-19-nor-D₃ acted after 1 α -hydroxylation by vitamin D 1 α -hydroxylase (CYP27B1). However, metabolic studies of 25(OH)-19-nor-D₃ using recombinant CYP27B1 revealed that 25(OH)-19-nor-D₃ was rarely subjected to 1 α -hydroxylation. Therefore, in this report, we attempted to clarify the mechanism of 25(OH)-19-nor-D₃ action in intact cells using PZ-HPV-7 prostate cells. After incubating the cells with 25(OH)-19-nor-D₃, eight metabolites of 24-hydroxylase (CYP24A1) were detected, whereas no products of CYP27B1 including 1 α ,25-dihydroxy-19-nor-vitamin D₃ (1 α ,25(OH)₂-19-nor-D₃) were found. Furthermore, the time-dependent nuclear translocation of vitamin D receptor (VDR) and the subsequent transactivation of *cyp24A1* gene in the presence of 25(OH)-19-nor-D₃ were almost identical as those induced by 1 α ,25(OH)₂-19-nor-D₃. These results strongly suggest that 25(OH)-19-nor-D₃ directly binds to VDR as a ligand and transports VDR into the nucleus to induce transcription of *cyp24A1* gene. In addition, knock down of *cyp27B1* gene did not affect the anti-proliferative activity of 25(OH)-19-nor-D₃, whereas knock down of VDR attenuated the inhibitory effect. Thus, our results clearly demonstrate that the anti-proliferative activity of 25(OH)-19-nor-D₃ is VDR dependent but 1 α -hydroxylation independent, suggesting that 25(OH)D₃ analogs such as 25(OH)-19-nor-D₃ could be attractive candidates for anticancer therapy.

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Introduction

1 α ,25-Dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃), the active form of vitamin D₃, is well known to regulate cell proliferation and differentiation, bone and calcium metabolism, and immune responses (Dusso *et al.* 2005). Because it can inhibit cancer cell growth, 1 α ,25(OH)₂D₃ has been used to treat cancer patients in clinical trials (Masuda & Jones 2006, Trump *et al.* 2010). However, systemic administration of 1 α ,25(OH)₂D₃ can cause hypercalcemia and hypercalciuria (Dusso *et al.* 2005). Thus, 1 α ,25(OH)₂D₃ is not suitable as a therapeutic agent for cancer treatment. Accordingly, the analogs that are less calcemic but exhibit potent anti-proliferative activity have potential as therapeutic agents. Among the analogs of 1 α ,25(OH)₂D₃, 19-nor-vitamin D compounds such as 1 α ,25-dihydroxy-19-nor-vitamin D₃ (1 α ,25(OH)₂-19-nor-D₃) and 1 α ,25-dihydroxy-19-nor-vitamin D₂

(1 α ,25(OH)₂-19-nor-D₂) have received most of the attention (Sicinski *et al.* 2007, Glebocka *et al.* 2009). 1 α ,25(OH)₂-19-nor-D analogs possess anti-proliferative activities similar to 1 α ,25(OH)₂D₃ (Chen *et al.* 2000), whereas the analog is less calcemic than 1 α ,25(OH)₂D₃, when administered systemically (Llach *et al.* 1998, Martin *et al.* 1998, Schwartz *et al.* 2005).

Genomic actions of 1 α ,25(OH)₂D₃ are mediated by the vitamin D receptor (VDR). On binding of the hormone to the receptor, VDR heterodimerizes with retinoid X receptor (RXR) and translocates into the nucleus (Racz & Barsony 1999, Prufer & Barsony 2002). The heterodimer associates with vitamin D-responsive elements in the promoter of vitamin D target genes such as 24-hydroxylase (*cyp24A1*; Zierold *et al.* 1995, Dusso *et al.* 2005). Some 19-nor-vitamin D analogs such as 1 α ,25(OH)₂-19-nor-D₃ also have affinity for VDR and may act through the cognate receptor (Urushino *et al.* 2007).

Since the discovery of 1 α ,25(OH)₂D₃, 1 α -hydroxylation is believed to be essential for the actions of 25-hydroxyvitamin D₃ (25(OH)D₃) and its analogs (Chen *et al.* 2000, Dusso *et al.* 2005). Hence, 25(OH)D₃ and its analogs are considered to be pro-bioactive substances. Indeed, LNCaP, a human prostate cancer cell line, whose proliferation is refractory to 25(OH)D₃, become responsive after transfection with the 1 α -hydroxylase (*cyp27B1*) cDNA plasmid (Whitlatch *et al.* 2002). Reduced CYP27B1 activity in human prostate cancer cells correlates with decreased susceptibility to 25(OH)D₃-induced growth inhibition (Hsu *et al.* 2001). We also reported that the growth of PZ-HPV-7 cells, an immortalized normal prostate cell line that expresses CYP27B1 (Wang *et al.* 2004), could be inhibited by not only 1 α ,25(OH)₂-19-nor-D₃ but also 25-hydroxy-19-nor-vitamin D₃ (25(OH)-19-nor-D₃; Arai *et al.* 2005). This finding led us to hypothesize that 25(OH)-19-nor-D₃ exerts its anti-proliferative activity after 1 α -hydroxylation mediated by CYP27B1.

The enzymatic properties of CYP enzymes involved in vitamin D₃ metabolism have been extensively studied and characterized in detail in our laboratory (Sakaki *et al.* 1999, 2000, 2005, Sawada *et al.* 2000, 2004). Using the reconstituted, cell-free system containing CYP24A1, CYP27A1, and CYP27B1, we have identified the metabolites of 19-nor-vitamin D₃ compounds. CYP24A1 catalyzes both C-23 and C-24 oxidation pathways and converts 1 α ,25(OH)₂-19-nor-D₃ into various metabolites such as 1 α ,24,25-trihydroxy-19-nor-vitamin D₃ (1 α ,24,25(OH)₃-19-nor-D₃) and 1 α ,23,25-trihydroxy-19-nor-vitamin D₃ (1 α ,23,25(OH)₃-19-nor-D₃; Urushino *et al.* 2007). We have also revealed that CYP27A1, which has subtle 25(OH)D₃ CYP27B1 activity, metabolizes 25(OH)-19-nor-D₃ into a single product, 25,26-dihydroxy-19-nor-vitamin D₃, which has almost no affinity for VDR (Urushino *et al.* 2007). The same product was also detected as a major metabolite by CYP27B1. To our surprise, 25(OH)-19-nor-D₃ was hardly converted to 1 α ,25(OH)₂-19-nor-D₃ by CYP27B1. The kinetic analysis of 1 α -hydroxylation of 25(OH)D₃ and 25(OH)-19-nor-D₃ demonstrated that the k_{cat}/K_m for 25(OH)-19-nor-D₃ is <0.1% than that for 25(OH)D₃ (Urushino *et al.* 2007). The extremely low activity toward 25(OH)-19-nor-D₃ could be explained by the docking model for CYP27B1. Although 25(OH)-19-nor-D₃ is hardly subjected to 1 α -hydroxylation, it inhibited prostate cell proliferation (Arai *et al.* 2005), suggesting that the anti-proliferative effects may be exerted without 1 α -hydroxylation.

In this study, we studied the metabolism and action of 25(OH)-19-nor-D₃ using human prostatic epithelial PZ-HPV-7 cells. Although eight 25(OH)-19-nor-D₃ metabolites catalyzed by CYP24A1 were detected, 1 α ,25(OH)₂-19-nor-D₃ was not. Nuclear translocation of VDR, transcriptional activation of CYP24A1, and

inhibition of cell proliferation by 25(OH)-19-nor-D₃ were also examined to confirm our hypothesis that 25(OH)-19-nor-D₃ has anti-proliferative activity in prostate cells without 1 α -hydroxylation.

Materials and methods

Chemical reagents

19-nor-Vitamin D₃ analogs (25(OH)-19-nor-D₃, 1 α ,25(OH)₂-19-nor-D₃) were synthesized as described previously (Arai *et al.* 2005). 25(OH)D₃ was purchased from Wako Pure Chemicals Co., Ltd (Osaka, Japan).

Cell culture

The transformed PZ-HPV-7 cell line (CRL-2221) was obtained from ATCC (Manassas, VA, USA). The cell line was derived from epithelial cells of the peripheral zone of the normal prostate tissue by transfecting with HPV18 DNA. PZ-HPV-7 cells were maintained in CnT-12, which was purchased from CELLnTEC Advanced Cell Systems (Bern, Switzerland) and is a fully defined, serum and bovine pituitary extract-free formulation. All media were supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen). All cells were grown in a 37 °C humidified incubator with an atmosphere of 5% CO₂.

Metabolism of 25(OH)-19-nor-D₃ using reverse-phase HPLC and liquid chromatography tandem mass spectroscopy

Cells, plated in a 35 mm dish at a density of 2×10^5 cells/well, were incubated with 1 μ M 25(OH)-19-nor-D₃ for 24 h. After the incubation, metabolites were extracted using chloroform/methanol (3:1, v/v) from a mixture of cells and medium. The organic phase was recovered and dried down under reduced pressure. The resultant residue was dissolved in acetonitrile and applied to HPLC under the following conditions: column, YMC-Pack ODS-AM (5 μ m; 4.6 $\times$ 300 mm; YMC Co., Kyoto, Japan); u.v. detection, 254 nm; flow rate, 1.0 ml/min; column temperature, 40 °C; mobile phase, linear gradient of 20–100% acetonitrile aqueous solution per 25 min followed by 100% acetonitrile for 20 min. Isolated metabolites from HPLC effluents were subjected to mass spectrometric analysis using a Finnegan Mat TSQ-70 with atmospheric pressure chemical ionization, positive mode. The conditions of liquid chromatography (LC) were as follows: column, reverse-phase ODS column (μ Bondapak C18, 5 μ m; Waters, Milford, MA, USA; 6 $\times$ 150 mm); mobile phase, 80% methanol aqueous solution per 25 min; flow rate, 1.0 ml/min; and u.v. detection, 254 nm (Urushino *et al.* 2007).

Immunofluorescence analysis

PZ-HPV-7 cells were seeded on coverslips and allowed to adhere overnight. After incubation with 100 nM 25(OH)-19-nor-D₃ or 1 nM 1 α ,25(OH)₂-19-nor-D₃ for 0–90 min, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.1% Tween-20/PBS, and blocked with 1% BSA. Cells were washed and incubated with antihuman VDR rat monoclonal antibody (Abcam, Cambridge, UK) for 2 h at room temperature. Then, cells were washed and incubated with Alexa-555-labeled anti-rat secondary antibody (Invitrogen) for 2 h. After staining nuclei with DAPI, cells were visualized using a fluorescence microscope (IX70; Olympus, Tokyo, Japan).

RNA isolation and real-time RT-PCR analysis

PZ-HPV-7 cells, plated in a 35 mm dish at a density of 2×10^5 cells/well, were treated with 25(OH)-19-nor-D₃ or 1 α ,25(OH)₂-19-nor-D₃ for 2–20 h. After the incubation, total RNA was isolated from PZ-HPV-7 using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. cDNAs were prepared using Prime Script RT reagent kit (TaKaRa, Otsu, Japan) from 1 μ g total RNA. The transcript levels were determined by real-time PCR using 7500 Real-Time RT-PCR System (Applied Biosystems, Carlsbad, CA, USA) using SYBR Premix EX Taq II (TaKaRa). The PCR primers used were as follows: CYP24A1, 5'-CGCCGATAGCAGAGCTTCAA-3' (forward) and 5'-GGTGCTGAGTGTAGCATCTCAAC-3' (reverse); VDR, 5'-AAGCTGAACCTTGCATGAGGAG-3' (forward) and 5'-GTCCTGATGGCCTCAATC-3' (reverse); CYP27B1, 5'-CTTGCGGACTGCTCACTG-3' (forward) and 5'-CGCAGACTACGTTGTTTCAGG-3' (reverse); GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGTGAAGACGCCAGTGGA-3' (reverse); GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGTGAAGACGCCAGTGGA-3' (reverse). The comparative threshold cycle values were used to determine the relative mRNA expression levels as described previously (Munetsuna *et al.* 2009). Real-time RT-PCR results were normalized by GAPDH expression levels.

Suppression of gene expression by small interfering RNAs

Duplex small interfering RNAs (siRNAs) for CYP27B1 and VDR (Stealth select RNAi) were purchased from Invitrogen. The sequences of the three sets of siRNAs were as follows: CYP27B1 siRNA: 1) 5'-AAAGAAUUUGGCU-CUGGGAACUGGG-3' and 5'-CCCAGUUCCAGAGC-CAAUUCUUU-3', 2) 5'-UAGGAUCUGGGCCAAAGCC-AUUUGC-3' and 5'-GCAAAUGGCUUUGGCCCAAGUC-3', 3) 5'-AAUGCAAACAUCUGGUCCAGUCUC-3'

and 5'-GAGACUGGGACCAGAUGUUUGCAUU-3'; VDR siRNA: 1) 5'-UUGCCAAACACUUCGAGCACAAGGG-3' and 5'-CCCUUGUGCUCGAAGUGUUUGGCAA-3', 2) 5'-UAGCAUUGAAGUGAAAGCCAGUGGC-3' and 5'-GC-CACUGGCUUUCACUUCAAUGCUA-3', 3) 5'-UUUGGAUGCUGUAACUGACCAGGUC-3' and 5'-GACCU-GGUCAGUUACAGCAUCCAAA-3'. Each set of duplex siRNAs was transfected into PZ-HPV-7 cells for 24 h using Lipofectamine RNAiMax according to the manufacturer's instructions before treating with 19-nor-vitamin D₃ analogs.

Western blot analysis

Proteins were separated on a 10% SDS-PAGE and transferred to nitrocellulose transfer membrane (GE Healthcare, Buckinghamshire, UK). The membrane was incubated with anti-VDR rat monoclonal antibody or anti-calnexin rabbit polyclonal antibody (Abcam). Calnexin, which is one of the housekeeping proteins, was used as loading control. HRP-conjugated anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-rabbit IgG (Cell Signaling Technology, Waltham, MA, USA) was used as the secondary antibody. Protein bands were visualized using ECL Plus western blotting detection reagent (GE Healthcare). The chemiluminescence of bands for VDR or calnexin was digitized using a LAS-1000 image analyzer (Fujifilm, Tokyo, Japan).

Cell proliferation assay

MTT reagent (Wako) was used for determining the number of viable cells in proliferation. Cells were seeded in 96-well plates (1500 cells/well). Cells were treated with 25(OH)-19-nor-D₃ (0.1–1000 nM) or 1 α ,25(OH)₂-19-nor-D₃ (0.1–100 nM) after preculture for 24 h. After 4 days, MTT reagent (5 mg/ml) was added to the cells. The cells were further incubated for 4 h at 37 °C and then treated with 0.04 M HCl/isopropanol (20 μ l). Optical absorbance at 570 nm was measured using a microplate reader (Varioskan; Thermo Scientific, MA, Waltham, USA). The effect of 25(OH)D₃ (100 nM) on cell proliferation was also analyzed by counting cell numbers using counters (Invitrogen).

Statistical analysis

The statistical significance of differences in the level of mRNA for CYP24A1 induced by the 19-nor-vitamin D₃ analogs was analyzed by two-way ANOVA. The effect of the 19-nor-vitamin D₃ analogs on cell growth was analyzed by one-way ANOVA. If differences were found to be significant, the analyses were followed by *post hoc t*-test with Bonferroni correction. Gene

suppression by siRNAs was analyzed by the Student's *t*-test. The criterion for significance was $P < 0.05$. All results were expressed as mean $\pm$ S.E.M.

Results

Metabolism of 25(OH)-19-nor-D₃ in PZ-HPV-7 cells

Cells were incubated with 25(OH)-19-nor-D₃ at a concentration of 1 μ M for 24 h. After the incubation, metabolites of 25(OH)-19-nor-D₃ were analyzed by reverse-phase HPLC and LC tandem mass spectroscopy (LC-MS). Figure 1 depicts the HPLC profiles showing that at least eight metabolites of 25(OH)-19-nor-D₃ were produced in PZ-HPV-7 cells. To determine the chemical structures of the metabolites, we collected the metabolites in the effluents from HPLC and subjected them to mass spectrometric analysis. Figure 2 shows mass spectra of the metabolites. These metabolites numbered 1, 2, 3, 5, and 7 are as follows: M1: m/z 420.83 (M+H), m/z 403.05 (M+H-H₂O), m/z 385.11 (M+H-2H₂O), and m/z 367.12 (M+H-3H₂O) of which are consistent with the assumption that M1 is 23,25,26(OH)₃-19-nor-D₃ (Fig. 2A); M2: m/z 401.04 (M+H-H₂O) and m/z 418.99 (M+H), which are consistent with the assumption that M2 is 24-oxo-23,25(OH)₂-19-nor-D₃ (Fig. 2B); M3: m/z 315.01 (M+H-H₂O), m/z 297.06 (M+H-2H₂O), m/z 332.99 (M+H), and m/z 278.99 (M+H-3H₂O) of which are consistent with the assumption that M3 is tetranor-23(OH)-19-nor-D₃ (Fig. 2C); M5: m/z 369.14 (M+H-2H₂O), m/z 387.07 (M+H-H₂O), m/z 404.8 (M+H), and m/z 351.16 (M+H-3H₂O) of which are consistent with the assumption that M5 is 24,25(OH)₂-19-nor-D₃ (Fig. 2D); M7: m/z 403.01 (M+H), m/z 385.13 (M+H-H₂O), and m/z 367.16

(M+H-2H₂O) of which are consistent with the assumption that M7 is 24-oxo-25(OH)-19-nor-D₃ (Fig. 2E). Based on our previous results, metabolites 4, 6, and 8 are believed to be 23,25(OH)₂-19-nor-D₃, 23-oxo-25(OH)-19-nor-D₃, and 25,26,27-trinor-24-ene-(OH)-19-nor-D₃, respectively, and are the products of CYP24A1 catalysis (Sakaki *et al.* 2000, Sawada *et al.* 2004, Urushino *et al.* 2007). Of particular interest is the fact that no 1 α ,25(OH)₂-19-nor-D₃ was detected at its retention time between M2+M3 and M4+M5 (Fig. 1), suggesting that 25(OH)-19-nor-D₃ may not be subjected to 1 α -hydroxylation in PZ-HPV-7 cells. This finding is consistent with our previous findings showing that CYP27B1 hardly converted 25(OH)-19-nor-D₃ into 1 α ,25(OH)₂-19-nor-D₃ (Urushino *et al.* 2007).

Based on these data, several metabolic pathways for 25(OH)-19-nor-D₃ in PZ-HPV-7 cells are proposed as shown in Fig. 3. It is noted that the metabolic pathways in prostate cells are quite similar to those observed in studies using recombinant human CYP24A1 expressed in *Escherichia coli* cells (Sakaki *et al.* 2000).

Translocation of VDR into the nucleus by 25(OH)-19-nor-D₃ in PZ-HPV-7 cells

It has been reported that the activated VDR heterodimerizes with RXR and translocates into the nucleus (Racz & Barsony 1999). Thus, we examined the nuclear translocation of VDR induced by 25(OH)-19-nor-D₃ and 1 α ,25(OH)₂-19-nor-D₃. In Fig. 4, the left panels show nuclear import of VDR after incubation with 1 nM 1 α ,25(OH)₂-19-nor-D₃ for 0–90 min. At 0 min, VDR appeared to be dispersed all over the cytoplasm. Thereafter, time-dependent nuclear translocation of VDR was observed at 30–60 min. Finally, at 90 min, most of VDR was localized in the nucleus. Rapid nuclear translocation of VDR was also observed by the addition of 100 nM 25(OH)-19-nor-D₃ (Fig. 4, right panels). Both 25(OH)-19-nor-D₃ and 1 α ,25(OH)₂-19-nor-D₃ showed similar time-dependent translocation (Fig. 4), suggesting that 25(OH)-19-nor-D₃ directly binds to VDR as a ligand to induce nuclear translocation without converting to its 1 α -hydroxylated metabolite.

Effect of 25(OH)-19-nor-D₃ on CYP24A1 mRNA level

It has been demonstrated that nuclear import of VDR is closely linked to the transcriptional activation (Racz & Barsony 1999). Because both 1 α ,25(OH)₂-19-nor-D₃ and 25(OH)-19-nor-D₃ induced nuclear translocation of VDR, transcriptional activation by VDR may occur. We analyzed CYP24A1 mRNA level, a well-known indicator of activated VDR (Zierold *et al.* 1995, Flanagan *et al.* 2006). PZ-HPV-7 cells were incubated with 1 α ,25(OH)₂-19-nor-D₃ (1 and 10 nM) or

Figure 1 HPLC profiles of 25(OH)-19-nor-D₃ and its metabolites in extracts of PZ-HPV-7 cells. After cells were incubated with 1 μ M 25(OH)-19-nor-D₃, the reaction mixture was extracted and analyzed by the reversed-phase HPLC. Numbers indicate the metabolites in the metabolic pathways in Fig. 3.

Figure 2 Mass spectra of 25(OH)-19-nor-D₃ metabolites shown in Fig. 1. M1 (A), M2 (B), M3 (C), M5 (D), and M7 (E) in Fig. 1 were isolated by HPLC and analyzed by mass spectrometry as described in the Materials and methods section.

25(OH)-19-nor-D₃ (10 and 100 nM) for 2–20 h. *CYP24A1* mRNA was quantified by real-time RT-PCR. As expected, a significant increase of *CYP24A1* mRNA level was observed at 2 h (Fig. 5, left panel). The *CYP24A1* mRNA level was increased as high as 700 times with 10 nM 1 α ,25(OH)₂-19-nor-D₃ at 5 h and then decreased. The *CYP24A1* mRNA level was also increased 150 and 800 times with 10 and 100 nM 25(OH)-19-nor-D₃, respectively, in the same time-dependent manner as 1 α ,25(OH)₂-19-nor-D₃.

Effect of 25(OH)-19-nor-D₃ on PZ-HPV-7 cell growth

It has been demonstrated that VDR activation is required for growth inhibition of prostate cell (Miller *et al.* 1992). It was also shown that 1 α ,25(OH)₂-19-nor-D₂ and 1 α ,25(OH)₂-19-nor-D₃ were as potent as

1 α ,25(OH)₂D₃ in inhibiting prostate cell proliferation (Chen *et al.* 2000, Chen & Holick 2003). Therefore, we investigated the effects of both 25(OH)-19-nor-D₃ and 1 α ,25(OH)₂-19-nor-D₃ on PZ-HPV-7 cell proliferation. PZ-HPV-7 cells were incubated with varying concentrations of 25(OH)-19-nor-D₃ (0.1–1000 nM) or

Figure 3 C-23 and C-24 oxidation pathways of 25(OH)-19-nor-D₃ in PZ-HPV-7 cells. Numbers indicate the metabolites shown in Figs 1 and 2.

Figure 4 Translocation of VDR into the nucleus in the presence of 1 nM 1 α ,25(OH)₂-19-nor-D₃ (left) or 100 nM 25(OH)-19-nor-D₃ (right). After cells were incubated with the 19-nor-vitamin D₃ analogs for 30–90 min, VDR was detected using a fluorescence microscope as described in the Materials and methods section.

1 α ,25(OH)₂-19-nor-D₃ (0.1–100 nM). Consistent with our previous reports, the cell growth was inhibited 40–80% by 1–100 nM 1 α ,25(OH)₂-19-nor-D₃ (Fig. 6, closed circles), whereas 100 and 1000 nM 25(OH)-19-nor-D₃ significantly decreased cell growth by 60–75% (Fig. 6, open circles) compared with the non-treated cells. As is the case with CYP24A1 transcriptional activity, the effect of 25(OH)-19-nor-D₃ on cell growth was 10–100 times lower than that of 1 α ,25(OH)₂-19-nor-D₃. For further clarification, we next examined whether 25(OH)-19-nor-D₃ had anti-proliferation activity using CYP27B1 siRNA- or VDR siRNA-transfected cells. Twenty-four hours after transfection with CYP27B1 siRNA, we incubated the cells for 4 days with 25(OH)-19-nor-D₃ (1000 nM) or 1 α ,25(OH)₂-19-nor-D₃ (100 nM). Incubation of CYP27B1 knockdown cells with 1000 nM 25(OH)-19-nor-D₃ or 100 nM 1 α ,25(OH)₂-19-nor-D₃ decreased cell growth (Fig. 7A and B, middle panels), which is not statistically different from the non-transfected cells (Fig. 7A and B left panels). However, transfection with VDR siRNA abolished the anti-proliferation activity of both 25(OH)-19-nor-D₃ and 1 α ,25(OH)₂-19-nor-D₃ (Fig. 7A and B, right panels).

Transfection with non-silencing siRNA did not affect cell growth compared to non-transfected cells. We confirmed that expression of CYP27B1 and VDR mRNA was significantly reduced compared with non-silencing siRNA-transfected cells (Fig. 7C). We also confirmed that VDR protein level was decreased by VDR siRNA transfection (Fig. 7C right panel). However, we have failed to detect CYP27B1 protein by western blot probably due to its low expression level in PZ-HPV-7 cells. Alternatively, we analyzed the effect of CYP27B1 siRNA transfection on the enzyme activity. We revealed that CYP27B1 enzyme activity using [H³]-25(OH)D₃ was reduced to about 20% with the CYP27B1 siRNA transfection in PZ-HPV-7 cells (data not shown).

These results strongly support our conclusion that 25(OH)-19-nor-D₃ induces cell growth inhibition mediated through VDR in PZ-HPV-7 cells without its conversion to 1 α ,25(OH)₂-19-nor-D₃ by CYP27B1.

Discussion

A large number of vitamin D analogs have been synthesized and studied for potential clinical application (Binderup *et al.* 1991, Bouillon *et al.* 1995, Yamada *et al.* 2003, Posner *et al.* 2010). Among vitamin D₃ analogs, it has been reported that A-ring-modified 19-nor-vitamin D compounds have unique biological activity and can alter the VDR-coactivator interaction, resulting in selective potentiation of the transcriptional function (Kittaka *et al.* 2000, Konno *et al.* 2000, Suhara *et al.* 2001, Ono *et al.* 2003, Arai *et al.* 2005). For example, 1 α ,25(OH)₂-19-nor-D₂ (also called paricalcitol or Zemplar) was approved by the Food and Drug Administration for the treatment of secondary hyperparathyroidism and clinical trials have shown that the compound is non-calcemic (Llach *et al.* 1998, Martin *et al.* 1998, Schwartz *et al.* 2005). 19-nor-Vitamin D₃

Figure 5 Effect of 1 α ,25(OH)₂-19-nor-D₃ or 25(OH)-19-nor-D₃ on CYP24A1 mRNA level. PZ-HPV-7 cells were incubated with 1 α ,25(OH)₂-19-nor-D₃ (1, 10 nM) (left) or 25(OH)-19-nor-D₃ (10, 100 nM) (right) for 2–20 h. The amounts of CYP24A1 mRNA were determined by real-time RT-PCR. The mRNA contents are given as values relative to those in vitamin D₃ incubated cells for 0 h. Error bars, s.e.m. The values are means of the results from four separate experiments.

Figure 6 Effect of 1 α ,25(OH)₂-19-nor-D₃ or 25(OH)-19-nor-D₃ on cell proliferation. Cell proliferation was analyzed using MTT assay. The cells were incubated with 1 α ,25(OH)₂-19-nor-D₃ (closed circles) or 25(OH)-19-nor-D₃ (open circles) for 4 days. Error bars, S.E.M. The values are means of the results from three separate experiments. * P < 0.05, ** P < 0.01, compared with control cells.

derivatives are also believed to be useful for prostate cancer chemoprevention and therapy (Chen *et al.* 2000, Rashid *et al.* 2001, Chen & Holick 2003, Arai *et al.* 2005). In this study, we clearly demonstrated that not only 1 α ,25(OH)₂-19-nor-D₃ but also 25(OH)-19-nor-D₃ has biological activity in human prostate cells. We would like to emphasize that a detailed metabolic analysis was linked to reveal genomic actions of the vitamin D analogs in this study. Based on our previous studies, all the metabolites detected in PZ-HPV-7 cells were produced by CYP24A1 catalysis. It should be noted that 1 α ,25(OH)₂-19-nor-D₃ was not found, which is consistent with our previous findings using a cell-free reconstituted system showing that CYP27B1 and CYP27A1 hardly converted 25(OH)-19-nor-D₃ into 1 α ,25(OH)₂-19-nor-D₃ (Urushino *et al.* 2007). The absence of a methylene group at C-19 position may prevent the binding of 19-nor compounds to the substrate-binding pocket of CYP27B1 without affecting their binding to CYP24A1. Generally, metabolites produced by CYP24A1 are likely to have reduced affinity for VDR compared with the substrate (Bouillon *et al.* 1995). Therefore, it seems likely that no metabolites more active than 25(OH)-19-nor-D₃ were produced in PZ-HPV-7 cells.

In this study, we concluded that 25(OH)-19-nor-D₃ itself exhibits its biological activity via binding to VDR, although the affinity of the compound for VDR is much lower than 1 α ,25(OH)₂-D₃. Our previous study demonstrated that the affinity of 1 α ,25(OH)₂-19-nor-D₃ is ~4% of 1 α ,25(OH)₂-D₃ (Urushino *et al.* 2007). In addition, based on the fact that the affinity of 25(OH)-D₃ is about 500 times less than that of 1 α ,25(OH)₂-D₃ (Bouillon *et al.* 1995), the affinity of 25(OH)-19-nor-D₃ appears to be about 100 times less than that of 1 α ,25(OH)₂-19-nor-D₃. Thus, the major reason why 25(OH)-19-nor-D₃ requires 10–100 times higher concentration than 1 α ,25(OH)₂-19-nor-D₃ to

achieve the same effects may be due to its much lower affinity for VDR comparing to 1 α ,25(OH)₂-19-nor-D₃.

It has been demonstrated that VDR translocates into the nucleus in a hormone-dependent manner (Racz & Barsony 1999). We examined the time course of VDR nuclear transport induced by 19-nor-vitamin D₃ analogs. Immunofluorescence analysis clearly demonstrated that 25(OH)-19-nor-D₃ acted as quickly as 1 α ,25(OH)₂-19-nor-D₃. These results lead us to conclude that 25(OH)-19-nor-D₃ binds to VDR directly, without being subjected to 1 α -hydroxylation.

Nuclear translocation of VDR into the nucleus within 90 min could explain a significant increase in *CYP24A1* mRNA level at 2 h. The time-dependent changes of

Figure 7 Effect of siRNA knock down of CYP27B1 and VDR knockdown on PZ-HPV-7 cell proliferation. Cells were transfected with *CYP27B1* siRNA or *VDR* siRNA for 24 h before 25(OH)-19-nor-D₃ or 1 α ,25(OH)₂-19-nor-D₃ treatment. No siRNA-, *CYP27B1* siRNA-, or *VDR* siRNA-transfected cells were incubated with 25(OH)-19-nor-D₃ (0, 1000 nM) (A) or 1 α ,25(OH)₂-19-nor-D₃ (0, 100 nM) (B). Transfection of *CYP27B1* siRNA decreased its mRNA level (C, left). Transfection of *VDR* siRNA decreased both its mRNA and protein levels (C, middle and right). Calnexin was used as an internal standard. The mRNA contents after the incubation are given as values relative to those in non-silencing siRNA-transfected cells. Error bars, S.E.M. The values are means of the results from at least three separate experiments. * P < 0.05, ** P < 0.01, compared with control cells.

Figure 8 The proposed mode of 25(OH)-19-nor-D₃ action based on this study. 25(OH)-19-nor-D₃ binds to VDR as a ligand to regulate genes such as *CYP24A1* and cell growth-related genes. 25(OH)-19-nor-D₃ was predominantly metabolized by CYP24A1 induced after the addition of 25(OH)-19-nor-D₃ without subjecting to 1α-hydroxylation to form 1α,25(OH)₂-19-nor-D₃.

CYP24A1 mRNA levels induced by 25(OH)-19-nor-D₃ and 1α,25(OH)₂-19-nor-D₃ are quite similar, demonstrating that the VDR-mediated transactivation is closely linked to ligand-dependent translocation of VDR. Based on our current metabolism analysis and previous enzymatic analysis (Urushino *et al.* 2007), the potential amount of 1α,25(OH)₂-19-nor-D₃ that could be produced from 100 nM 25(OH)-19-nor-D₃ may be <0.01 nM. The transcriptional activation of *CYP24A1* by 0.01 nM 1α,25(OH)₂-19-nor-D₃ is negligible (data not shown). These results strongly suggest that 25(OH)-19-nor-D₃ binds to VDR as a ligand and induces nuclear translocation of VDR to activate transcription of *cyp24a1* gene.

1α,25(OH)₂D₃ and its 19-nor analogs have growth inhibitory effect on prostate cells (Miller *et al.* 1992, Chen *et al.* 2000, Chen & Holick 2003). In this study, we confirmed that not only 1α,25(OH)₂-19-nor-D₃ but also 25(OH)-19-nor-D₃ inhibited cell growth. The anti-growth effect of 25(OH)-19-nor-D₃ and 1α,25(OH)₂-19-nor-D₃ was attenuated by knock down of *VDR* gene, implying that the effect depends on VDR. However, it should be noted that the anti-proliferative activity of 25(OH)-19-nor-D₃ was not affected by knock down of *cyp27b1* gene. Taken together, we conclude that the mode of 25(OH)-19-nor-D₃ action derived from this study is VDR dependent but *CYP27B1* independent.

Anti-proliferative activity of vitamin D₃ and its analogs is well known to be correlated with an increase in the levels of the cyclin-dependent kinase inhibitors (CKI) such as p21^{waf1,CIP1} and p27^{Kip1}, leading to a profound decrease in cyclin-dependent kinase 2 activity and inhibition of cell cycle progression from G₁ to S phase in LNCaP prostate cancer cells (Yang & Burnstein 2003). Recently, it has been demonstrated that cystatin D, an inhibitor of several cysteine proteases

of the cathepsin family, is a tumor suppressor gene induced by 1α,25(OH)₂D₃ (Alvarez-Diaz *et al.* 2009). Anti-proliferative effect of 25(OH)-19-nor-D₃ could be mediated by CKI and/or cystatin D. Further study is necessary to clarify the mode of 25(OH)-19-nor-D₃ action.

Based on the present results, we propose a novel action model of 25(OH)-19-nor-D₃ as shown in Fig. 8. This model shows a direct action of 25(OH)-19-nor-D₃ as a ligand of VDR and differs from a classical model that 25(OH)D₃ analogs exert its biological function after 1α-hydroxylation by CYP27B1. It should be noted that the model in Fig. 8 resembles the action model proposed by Tuohimaa's group, suggesting a unique biological activity of 25(OH)D₃ as a ligand of VDR in human prostate cells (Lou *et al.* 2004, 2010). Recently, Deluca *et al.* (2011) also suggested a direct action of 25(OH)D₃ as a VDR ligand in *Cyp27b1* knockout mice. In this study, we also examined the effect of 25(OH)D₃. The addition of 100 nM 25(OH)D₃ to no siRNA- and *CYP27B1* siRNA-transfected PZ-HPV-7 cells decreased cell growth to 37 and 45%, respectively. However, VDR knock down canceled the anti-proliferative activity of 25(OH)D₃. These results strongly suggest a direct action of 25(OH)D₃ as a VDR ligand. The genomic actions of the vitamin D compounds might not require the hydroxylation at C-1 position in human prostate cells. As *CYP27A1* and *CYP27B1* have been shown to be expressed in prostate tissue (Flanagan *et al.* 2006), the autocrine regulation of the two vitamin D 25-hydroxylases could play an important role in prostate cancer prevention. The action model of 25(OH)-19-nor-D₃ shown in Fig. 8 suggests that 25(OH)D₃ analogs, such as 25(OH)-19-nor-D₃, are attractive candidates for anticancer drugs with much less calcemic activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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