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

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Abstract

According to the prevailing paradigm, 1α-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 1α-hydroxylation by vitamin D 1α-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 1α-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 25(OH)-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 1α,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 cyp24A1 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 1α-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

1α,25-Dihydroxyvitamin D3 (1α,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, 1α,25(OH)2D3 has been used to treat cancer patients in clinical trials (Masuda & Jones 2006, Trump et al. 2010). However, systemic administration of 1α,25(OH)2D3 can cause hypercalcemia and hypercalciuria (Dusso et al. 2005). Thus, 1α,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 1α,25(OH)2D3, 19-nor-vitamin D compounds such as 1α,25-dihydroxy-19-nor-vitamin D3 (1α,25(OH)2-19-nor-D3) and 1α,25-dihydroxy-19-nor-vitamin D2 (1α,25(OH)2-19-nor-D2) have received most of the attention (Scimsky et al. 2007, Glebocka et al. 2009). 1α,25(OH)2-19-nor-D analogs possess anti-proliferative activities similar to 1α,25(OH)2D3 (Chen et al. 2000), whereas the analog is less calcemic than 1α,25(OH)2D3, when administered systemically (Llach et al. 1998, Martin et al. 1998, Schwartz et al. 2005).

Genomic actions of 1α,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 1α,25(OH)2-19-nor-D also have affinity for VDR and may act through the cognate receptor (Urushio et al. 2007).
Since the discovery of 1α,25(OH)2D3, 1α-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-apoptotic substances. Indeed, LNCaP, a human prostate cancer cell line, whose proliferation is refractory to 25(OH)D3, become responsive after transfection with the 1α-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 1α,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 1α-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 1α,25(OH)2-19-nor-D3 into various metabolites such as 1α,24,25-trihydroxy-19-nor-vitamin D3 (1α,24,25(OH)3-19-nor-D3) and 1α,23,25-trihydroxy-19-nor-vitamin D3 (1α,23,25(OH)3-19-nor-D3; Urushino et al. 2007). We have also revealed that CYP27B1, 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 1α,25(OH)2-19-nor-D3 by CYP27B1. The kinetic analysis of 1α-hydroxylation of 25(OH)D3 and 25(OH)-19-nor-D3 demonstrated that the kcat/Km 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 1α-hydroxylation, it inhibited prostate cell proliferation (Arai et al. 2005), suggesting that the anti-proliferative effects may be exerted without 1α-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, 1α,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 1α-hydroxylation.

### Materials and methods

#### Chemical reagents

19-nor-Vitamin D3 analogs (25(OH)-19-nor-D3, 1α,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 µg/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 spectrometry

Cells, plated in a 35 mm dish at a density of 2×105 cells/well, were incubated with 1 µM 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 µm; 4.6×300 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 (μBondapak C18, 5 µm; Waters, Milford, MA, USA; 6×150 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 1z,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 × 10⁵ cells/well, were treated with 25(OH)-19-nor-D₃ or 1z,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 μg 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'-CGCCGAT-TAGCAGAGCTTCAA-3' (forward) and 5'-GGTGCTTGTGATGTAGCATCTCAAC-3' (reverse); VDR, 5'-AAGCT-GAACCTTGCACTGGAGG-3' (forward) and 5'-GTCCGT-GATGCGCTCAATC-3' (reverse); CYP27B1, 5'-GGTCCGT-GAAGCTTGCTCAACTG-3' (forward) and 5'-GGCCGGT-GGTGCGCTCA-3' (reverse); GAPDH, 5'-GCCGAGCAGCTGCTGCAAT-3' (forward) and 5'-GGTGCCTG-GAAGCTAAG-3' (reverse); CYP24A1, 5'-GGCCCGAGCAGCTGCTGCAAT-3' (forward) and 5'-GGTGCCTG-GAAGCTAAG-3' (reverse).

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'-AAAGAAUUUUGCU-CUGGGAACUGGG-3' and 5'-CCCAGUUCUCAGACGCAAUUUU-3'; 2) 5'-UGAAUUGGCAAGCAAGCG-3' and 5'-GCCUGGUACUUCAGACGCAAUUUU-3'; 3) 5'-AAUGUAUCUGGCUUCAGACGCAAUUUU-3'.

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-caldesmon rabbit polyclonal antibody (Abcam). Caldesmon, which is one of the housekeeping proteins, was used as loading control. HRP-conjugated anti-rabbit 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 caldesmon 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 1z,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 μl). 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 ± 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 μM 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 387.06 (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 369.14 (M + H − H2O), m/z 351.16 (M + H), and m/z 351.16 (M + H − 3H2O) of which are consistent with the assumption that M3 is tetrano-23(OH)-19-nor-D3 (Fig. 2C); M5: m/z 367.16 (M + H − 2H2O), 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 1α,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 1α-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 1α,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 1α,25(OH)2-19-nor-D3. In Fig. 4, the left panels show nuclear import of VDR after incubation with 1 nM 1α,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 1α,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 1α-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 1α,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 1α,25(OH)2-19-nor-D3 (1 and 10 nM) or
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 1α,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 1α,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 1α,25(OH)2-19-nor-D2 and 1α,25(OH)2-19-nor-D3 were as potent as 1α,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 1α,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

![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.](www.endocrinology-journals.org)

Figure 2

![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.](www.endocrinology-journals.org)

Figure 3
1α,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 1α,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 1α,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 1α,25(OH)2-19-nor-D3 (100 nM). Incubation of CYP27B1 knockdown cells with 1000 nM 25(OH)-19-nor-D3 or 100 nM 1α,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 1α,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 1α,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, 1α,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
The cells were incubated with 1α,25(OH)_{2}-19-nor-D_3 (closed circles) or 25(OH)-19-nor-D_3 (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.

Figure 6 Effect of 1α,25(OH)_{2},19-nor-D_3 or 25(OH)-19-nor-D_3 on cell proliferation. Cell proliferation was analyzed using MTT assay. The cells were incubated with 1α,25(OH)_{2},19-nor-D_3 (closed circles) or 25(OH)-19-nor-D_3 (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.

In this study, we clearly demonstrated that not only 1α,25(OH)_{2},19-nor-D_3 but also 25(OH)-19-nor-D_3 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 1α,25(OH)_{2},19-nor-D_3 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-D_3 into 1α,25(OH)_{2},19-nor-D_3 (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-D_3 were produced in PZ-HPV-7 cells.

In this study, we concluded that 25(OH)-19-nor-D_3 itself exhibits its biological activity via binding to VDR, although the affinity of the compound for VDR is much lower than 1α,25(OH)_{2}D_3. Our previous study demonstrated that the affinity of 1α,25(OH)_{2},19-nor-D_3 is ~4% of 1α,25(OH)_{2}D_3 (Urushino et al. 2007). In addition, based on the fact that the affinity of 25(OH)-19-nor-D_3 is about 500 times less than that of 1α,25(OH)_{2}D_3 (Bouillon et al. 1995), the affinity of 25(OH)-19-nor-D_3 appears to be about 100 times less than that of 1α,25(OH)_{2},19-nor-D_3. Thus, the major reason why 25(OH)-19-nor-D_3 requires 10–100 times higher concentration than 1α,25(OH)_{2},19-nor-D_3 to achieve the same effects may be due to its much lower affinity for VDR comparing to 1α,25(OH)_{2},19-nor-D_3.

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 D analogs. Immunofluorescence analysis clearly demonstrated that 25(OH)-19-nor-D_3 acted as quickly as 1α,25(OH)_{2},19-nor-D_3. These results lead us to conclude that 25(OH)-19-nor-D_3 binds to VDR directly, without being subjected to 1α-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-D_3 or 1α,25(OH)_{2},19-nor-D_3 treatment. No siRNA, CYP27B1 siRNA-, or VDR siRNA-transfected cells were incubated with 25(OH)-19-nor-D_3 (0, 1000 nM) (A) or 1α,25(OH)_{2},19-nor-D_3 (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.
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 CYP2R1 have been shown to be expressed in prostate tissue (Flanagan et al. 2000, Chen & Holick 2003), the autocontrol 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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