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Original article

Synthesis and biological evaluation of 1α,25-dihydroxyvitamin D_3 analogues with aromatic side chains attached at C-17

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ABSTRACT

Two new analogues of the steroid hormone 1α,25-dihydroxyvitamin D_3 with aromatic side chains attached at C-17 were designed to investigate their effects on VDR, HL-60 cell differentiation and tumor cell proliferation. These analogues were prepared by the classical photochemical ring opening approach. After the protection of both the 1α- and 3β-hydroxyl in 1α-hydroxydehydroepiandrosterone with TBS groups, followed by bromination with NBS and debromination in the presence of γ-collidine, the diene intermediate was obtained. Hydrazone formation followed by iodine oxidation gave a vinyl iodide. The aromatic side chain at C-17 was introduced via the Negishi coupling of the resulting intermediate with an in situ generated zinc reagent with the substituted aryl bromide (CD-side chain) in the presence of catalytic amount of $Pd(PPh_3)_4$. After the removal of the TBDMS and MOM protective groups, followed by UV irradiation and the subsequent thermal reaction, the 1α,25-$(OH)_2$-D_3 analogues with a substituted phenyl ring attached at C-17 to replace the C-20 and C-21 were prepared. In the VDR competitive binding assay, compounds **2** and **3** almost lost their binding ability, and were only 0.01% and 0.015% as potent as the 1α,25-dihydroxyvitamin D_3. However, compounds **2** and **3** were as potent as 1α,25-$(OH)_2$-D_3 in inducing HL-60 cell differentiation at concentrations of 30, 100, 300, 1000 nM, respectively. Moreover, compounds **2** and **3** exhibited similar or better antiproliferative potency against MCF-7 human breast cancer cells, the IC_{50} values for analogues **2**, **3** and the natural hormone were 7.08, 7.56, and 12.5 μM, respectively.

1. Introduction

The hormonal metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3 or 1,25D] or calcitriol (**1**) (Fig. 1), initiates numerous biological functions required for human health. Besides its typical role in calcium and phosphorus homeostasis, calcitriol also regulates cell growth, differentiation, apoptosis, and adaptive/innate immune responses through the rapid activation of signal transduction pathways as well as the classic transcriptional activation pathways that require the nuclear vitamin D receptor (VDR) [1–3]. 1α,25-$(OH)_2$-D_3 and its analogues have been used in the clinic for the treatment of psoriasis, renal osteodystrophy, osteoporosis, secondary hyperparathyroidism, and vitamin D-resistant rickets. A number of 1α,25-$(OH)_2$-D_3 analogues also show extremely promising prospects for the treatment of cancer, AIDS, rheumatoid arthritis, inflammatory bowel diseases, type 1 diabetes, and Alzheimer's disease [4–6]. However, the use of calcitriol as a drug to treat hyperproliferative disorders is limited by its undesired hypercalcemic side effects [7]. Therefore, extensive efforts have been made in the design of novel 1α,25-$(OH)_2$-D_3 analogues that could dissociate between antiproliferative and/or prodifferentiating action and calcemic effects. Modifications on the A and/or CD rings or the aliphatic chain of the natural ligand have led to some potent vitamin D analogues that mediate transcriptional activity with a magnitude at least 10-fold higher than the natural ligand with identical or lower calcemic properties [8–14]. For example, the inversion of the configuration at C-20 usually resulted in increased activity both in vivo and in vitro [15]. The replacement of C-21 methyl group with a cyclopropyl ring at C-20 of 1α,25-$(OH)_2$-D_3 reduced the binding affinity for VDR and DBP, slightly improved

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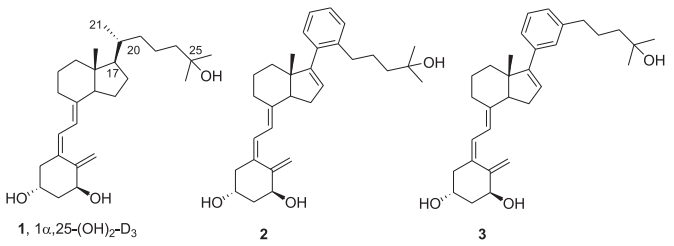


Fig. 1. 1α,25-$(OH)_2$-D_3 (**1**) and its analogues **2** and **3**.

the transactivational activity, and lowered the calcemic effects by 10-fold [16,17]. 1α,25$(OH)_2$-16-ene-20-cyclopropylvitamin D_3 proved to be several fold more potent than the natural hormone 1α,25-$(OH)_2$-D_3 as an anti-inflammatory agent [18]. Okamura's group reported a series of 1α,25-$(OH)_2$-D_3 analogues incorporating a phenyl ring at the aliphatic side chains, and found some analogues were just as potent as 1α,25-$(OH)_2$-D_3 in inducing HL-60 leukemic cell differentiation or inhibiting its proliferation, but were no toxic to the proliferation of normal human myeloid stem cells [19]. Many other side chain modifications and/or 16-ene introduction also resulted in a number of 1α,25-$(OH)_2$-D_3 analogues that exhibited more potent pro-differentiation, antiproliferative effects and transactivation potency, and some of them have the potential as anticancer agents [20–26]. Here we first report the synthesis and biological evaluations of two new vitamin D analogues **2** and **3** (Fig. 1) with a substituted phenyl ring attached at C-17 to replace the C-20 and C-21. Compared with the natural 1α,25-$(OH)_2$-D_3, this modification leads to the loss of two chiral centers at C-17 and C-20 in molecules **2** and **3**.

2. Chemistry

The classical strategy, including photo-reaction of provitamin D_3 into previtamin D_3 and the subsequent thermal conversion of previtamin D_3 into vitamin D_3, was applied for the synthesis of analogues **2** and **3**. To achieve this purpose, we first built the aromatic side chains through a new synthetic route. As shown in Scheme 1, the commercial available alcohol, **4a** or **4b**, was first converted to an iodide, which was reacted with the sodium salt of diethyl malonate to give a diester. Upon hydrolysis, compounds **5a** and **5b** were obtained in 63% and 65% yields, respectively. Decarboxylation and subsequent esterification produced the ethyl esters **6a** and **6b** in excellent yields. Coupling reaction of **6a** or **6b** with methylmagnesium bromide gave the tertiary alcohol, which was treated with chloromethyl methyl ether (MOMCl) to furnish the target aromatic side chains **7a** or **7b** in good yield.

1α-Hydroxydehydroepiandrosterone **8** (Scheme 2) was prepared from commercial available dehydroepiandrosterone by a modified procedure developed by our group, applying recoverable Pd/C catalyst mediated dehydrogenation of sterols as a key step [27]. After the TBDMS ether formation to protect the diols, diene **9** was obtained via sequential treatment with NBS and γ-collidine [28]. Diene **9** was then reacted with hydrazine hydrate in the presence of hydrazine sulfate to give hydrazone **10** in 87% yield. Oxidation of hydrazone **10** with iodine in the presence of a hindered guanidine base yielded the vinyl iodide **11**. The iodide **11** was instable when exposed to air, so it was used for the next reaction without further purification. Attaching the aromatic side chains to the C-17 position was successfully achieved through the Negishi coupling approach [29]. Bromide **7a** or **7b** was treated with *n*-butyllithium at −78 °C and then with zinc chloride in THF solution, the resulting zinc derivative was allowed to react with a preformed mixture of iodide **11** and $Pd(PPh_3)_4$ to give compounds **12a** and **12b**, which were treated sequentially with TBAF (tetrabutylammonium fluoride) and TsOH (*p*-toluenesulfonic acid) to remove the TBDMS and MOM protective groups to give the provitamins **13a** and **13b** in 31% and 30% yields (from **10**), respectively. Finally, these provitamins were converted to the desired vitamin D analogues **2** and **3** by the biomimetic sequence of photoirradiation with a high-pressure mercury lamp, followed by thermal isomerization and HPLC purification, in 16% and 14% yields, respectively.

3. Pharmacology

3.1. Competitive VDR binding assay

To examine the binding affinity of compounds **2** and **3** to the VDR, competitive VDR binding assay using bovine thymus was carried out by the standard method [30]. The relative potency of the analogues was calculated from the concentration required to displace 50% of the [^{3}H]-1α,25-dihydroxyvitamin D_3 from the receptor compared with the activity of 1α,25-dihydroxyvitamin D_3, which was assigned as 100 by definition.

3.2. Cell proliferation assay

The ability of compounds **2** and **3** to inhibit the proliferation of human MCF-7 breast cancer cells was examined by the conventional MTT assay. MCF-7 cells were incubated with compounds **2** and **3** at the indicated concentrations for 72 h, and their inhibiting rates were determined. 1α,25-Dihydroxyvitamin D_3 was used as a reference drug.

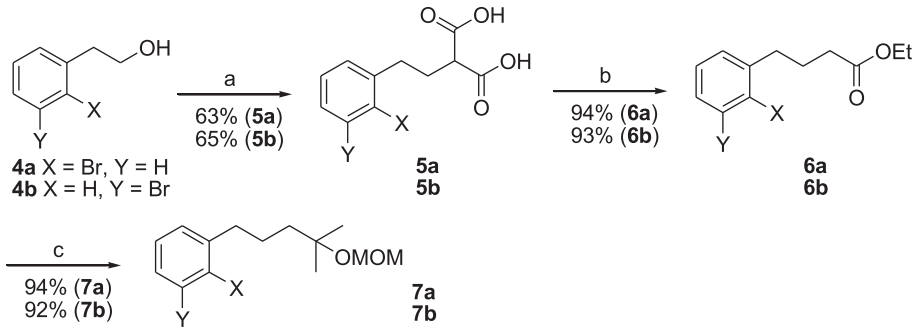
3.3. HL-60 cell differentiation assay

To evaluate the effects of compounds **2** and **3** on cell differentiation, HL-60 cells were incubated and treated with compound **2**, **3** or calcitriol (**1**) for 48 h at the indicated concentrations. 0.1% DMSO was used as vehicle. After the treatment, cells were transferred to slides with cytospin and applied for Wright's–Giemsa staining [31]. Forty microscopic view fields were analyzed and photographed.

4. Results and discussion

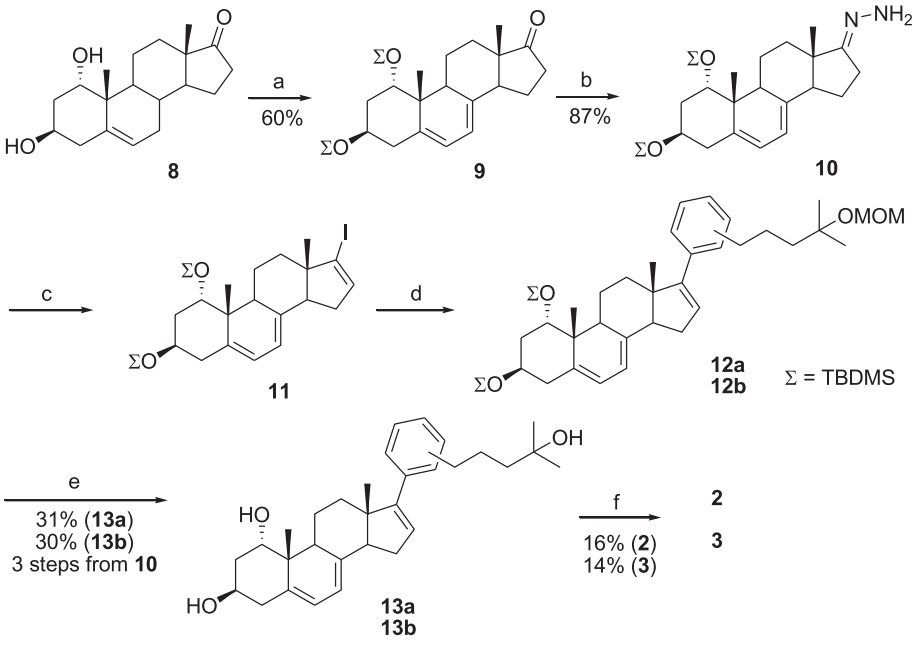
1α,25-Dihydroxyvitamin D_3 functions as the ligand for VDR, with the hormone–receptor complex inducing calcemic and phosphatemic effects that result in normal bone mineralization and remodeling. In the VDR binding assay, compounds **2** and **3** exhibited very low VDR affinity (see Supplementary data). They were comparable in potency to that of 25-OH-D_3, but were only 0.01% and 0.015% as potent as the parent 1α,25-$(OH)_2$-D_3 (Table 1). It is apparent that introducing a phenyl ring at the C-17 has much influence on the binding ability to the VDR.

X-ray crystal structure of Moras VDR(LBD)-1,25D complex was used for molecular docking studies by Sybyl–x1.3 software (PDB code: 1DB1) [32]. Docking analysis of compounds **2** and **3** demonstrated that the A, seco-B, C, and D rings present conformations that are similar to those observed in the presence of the natural ligand (Fig. 2). The A-ring hydroxyl groups make the same hydrogen bonds as the hVDR LBD bound to 1α,25-$(OH)_2$-D_3 complex, 1-OH with Ser237 and Arg274, 3-OH with Tyr143 and Ser278. However, the side chain tertiary hydroxyl group in compounds **2** and **3** form hydrogen bonding with His397, while the natural 25-OH binds to His305. In addition, the phenyl ring at the C-17 makes the side chain longer than that of natural hormone. All these



Reagents and conditions: (a) (i) I_2, PPh_3, Imidazole, CH_2Cl_2; (ii) NaH, $CH_2(CO_2Et)_2$, THF; (iii) KOH, EtOH, reflux; (b) (i) 170 °C, 30 min; (ii) $SOCl_2$, EtOH; (c) (i) CH_3MgBr, THF; (ii) MOMCl, DIEA, CH_2Cl_2.

Scheme 1. Synthesis of the aromatic side chains **7a** and **7b**.



Reagents and conditions: (a) (i) TBDMSCl, imidazole; (ii) NBS, γ-collidine; (b) N_2H_4•H_2O, N_2H_4•H_2SO_4; (c) I_2, THF, $(Me_2N)_2C$=NH; (d) **7a** or **7b**, *n*-BuLi, $ZnCl_2$, THF; **11**, $Pd(PPh_3)_4$, THF; (e) (i) TBAF,THF; (ii) TsOH, MeOH; (f) hν, THF; then, reflux.

Scheme 2. Synthesis of 1α,25-$(OH)_2$-D_3 analogues **2** and **3**.

factors might play important roles in reducing the binding affinity of compounds **2** and **3** to VDR.

Despite their low VDR binding affinity, compounds **2** and **3** were also as potent as the native 1α,25-$(OH)_2$-D_3 at the concentrations of 1.25, 2.5, 5.0 μM in the inhibition of the proliferation of human MCF-7 breast cancer cells, but were slightly more potent than 1α,25-$(OH)_2$-D_3 at the relative high concentrations of 10, 20, 40 μM (Fig. 3). In addition, the IC_{50} values for compounds **2**, **3** and 1α,25-$(OH)_2$-D_3 were 7.08, 7.56 and 12.5 μM, respectively (Table 1).

In the HL-60 cell differentiation inducing assay, compounds **2** and **3** were as potent as 1α,25-$(OH)_2$-D_3 in inducing HL-60 cell differentiation at concentrations of 30, 100, 300, 1000 nM, respectively. As shown in Fig. 4, the vehicle-treated HL-60 cells showed morphologic features of blast cells, including high nuclear cytoplasmic ratio, one or two nucleoli, non-condensed nuclear chromatin, deep stained cytoplasm. However, compounds **2**, **3** or 1α,25-$(OH)_2$-D_3 treatment led to maturing morphological changes, including decreased ratio of nuclear to cytoplasmic, reduced or disappeared nucleolus, condensed chromatin as well as lobed nuclei.

5. Conclusion

In summary, incorporating a phenyl ring at C-17 to replace the C-20 and C-21 of the native 1α,25-$(OH)_2$-D_3 made analogues **2** and **3** almost lose their VDR binding ability, but maintain their antiproliferative and prodifferentiating potency, indicating that these compounds may have therapeutic potential for the treatment of different hyperproliferative disorders.

Table 1
Results of VDR binding and MCF-7 cell proliferation assay.

Compds	VDR	MCF-7 (IC_{50}: μM)
2	0.01	7.08 ± 0.25
3	0.015	7.56 ± 0.28
1α,25-$(OH)_2$-D_3	100	12.5 ± 0.19
25-OH-D_3	0.01	–

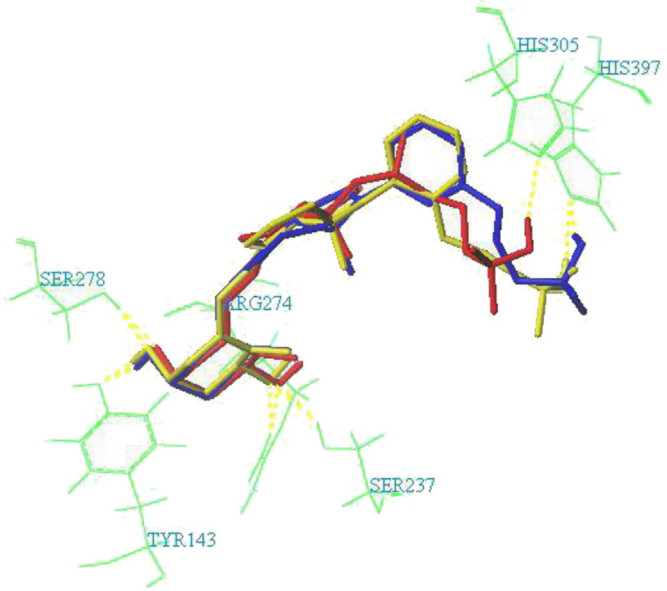


Fig. 2. Predicted conformations and binding modes of analogues **2** (yellow), **3** (blue) and 1α,25-$(OH)_2$-D_3 (red) in the VDR LBP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

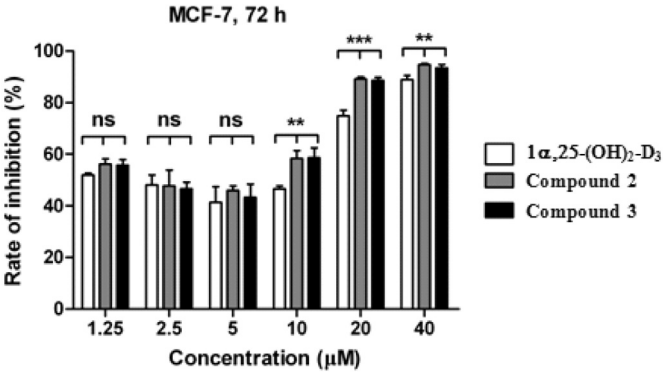


Fig. 3. The inhibition of MCF-7 human breast cancer cells at different concentrations. Error bars represent standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

6. Experimental section

6.1. Chemistry

Melting points (uncorrected) were determined on an X-6 micro-melting point apparatus (Beijing Tech. Co., Ltd.) and were uncorrected. UV data were recorded on a UV-2550 spectrophotometer (Shimadzu, Japan). NMR spectra were recorded on Bruker Avance DRX-600 or Varian INOVA 600 spectrometers operating at 600 (^{1}H) and 150 or 100 (^{13}C) MHz with TMS as internal standard. HRESIMS were carried out on an LTQ-Orbitrap XL. ESI-MS spectra were recorded on an API 4000 spectrometer. Column chromatography was performed on silica gel (300–400 mesh; Anhui Liangchen Silicon Material Co. Ltd., Anhui, PR China). All reactions involving oxygen- or moisture-sensitive compounds were carried out under a dry nitrogen atmosphere. Unless otherwise noted, reagents were added by syringe. THF was distilled from sodium/benzophenone immediately prior to use. All tested compounds are >98.5% pure by HPLC analysis, performed on a Shimadzu LC-20AT instrument, using the *ProntoSIL Eurobond* C18 (250 mm × 4.6 mm) or Agilent Eclipse XDB-C18 (150 × 4.6 mm) column.

6.1.1. 2-[2-(2-Bromophenyl)ethyl]malonic acid (**5a**)

To a solution of alcohol **4a** (15.0 g, 74.4 mmol) in dichloromethane (225 mL) was added triphenylphosphine (24.9 g, 94.9 mmol) and imidazole (6.45 g, 94.7 mmol). After stirring at room temperature for 20 min, iodine (22.5 g, 88.7 mmol) was added and the resulting dark brown mixture was stirred for overnight at 0 °C. A saturated solution of sodium thiosulfate (50 mL) was added and the mixture was allowed to warm to room temperature. The reaction mixture was extracted with dichloromethane, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduce pressure. Hexane was added to the residue and the white precipitate was filtered off. The filtrate was concentrated to give the crude 2-bromophenethyl iodide (23.0 g), which was used without further purification.

To a solution of ethyl malonate (295 mL) was slowly added NaH (12.0 g, 30.0 mmol) at 0 °C. After stirring for 30 min, a solution of the crude 2-bromophenethyl iodide (23.0 g) in THF (100 mL) was added. The reaction mixture was stirred at room temperature for overnight. After the completion of the reaction indicated by TLC, aqueous NH_4Cl (50 mL) was added. The resulting mixture was extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography on silica gel (hexane/AcOEt = 50:1) to give the substituted ethyl malonate (17.0 g) as a colorless oil, which was dissolved in 40 mL of 50% aqueous ethanol. To this solution, 9.30 g (165.8 mmol) of potassium hydroxide was added. After refluxing for 6 h, the reaction mixture was diluted with water and acidified with 15% hydrochloric acid solution. A colorless precipitate was collected to afford **5a** (13.5 g) in a total yield of 63% from **5a**. Mp: 157–158 °C. This data is in agreement with the reported value (156–158 °C) [33].

6.1.2. 2-[2-(3-Bromophenyl)ethyl]malonic acid (**5b**)

This compound was prepared from **4b** by the same procedure as described for compound **5a**. Yield: 65%; Mp: 161–163 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.36 (s, 1H), 7.32–7.34 (m, 1H), 7.13–7.17 (m, 2H), 3.37 (t, 1H, J = 7.2 Hz), 1.67–2.70 (m, 2H), 2.33–2.26 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 170.7, 144.0, 131.1, 130.6, 129.0, 127.5, 121.7, 51.0, 32.3, 30.0; HRMS (ESI): Calcd for $C_{11}H_{11}BrO_4$ $[M+Na]^+$ 308.9738 and 310.9718, found $[M+Na]^+$ 308.9752 and 310.9730.

6.1.3. Ethyl 4-(2-bromophenyl)butanoate (**6a**)

Acid **5a** (18.0 g, 62.7 mmol) was heated at 170 °C for 30 min until the evolution of carbon dioxide ceased. Ethanol (152 mL) was added, followed by dropwise addition of thionyl chloride (4.9 mL, 67.5 mmol). After stirring at room temperature for 3 h, the solvent was evaporated in vacuo. The residue was extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography on silica gel (hexane/AcOEt = 10:1) to give **6a** (16.0 g) as a colorless oil. Yield: 94%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.52 (dd, 1H, J = 7.8 Hz, 2.4 Hz), 7.21–7.24 (m, 2H), 7.04–7.07 (m, 1H), 4.11–4.15 (m, 2H), 2.76–2.79 (m, 2H), 2.34–2.37(m, 2H), 1.93–1.99 (m, 2H), 1.25–1.27

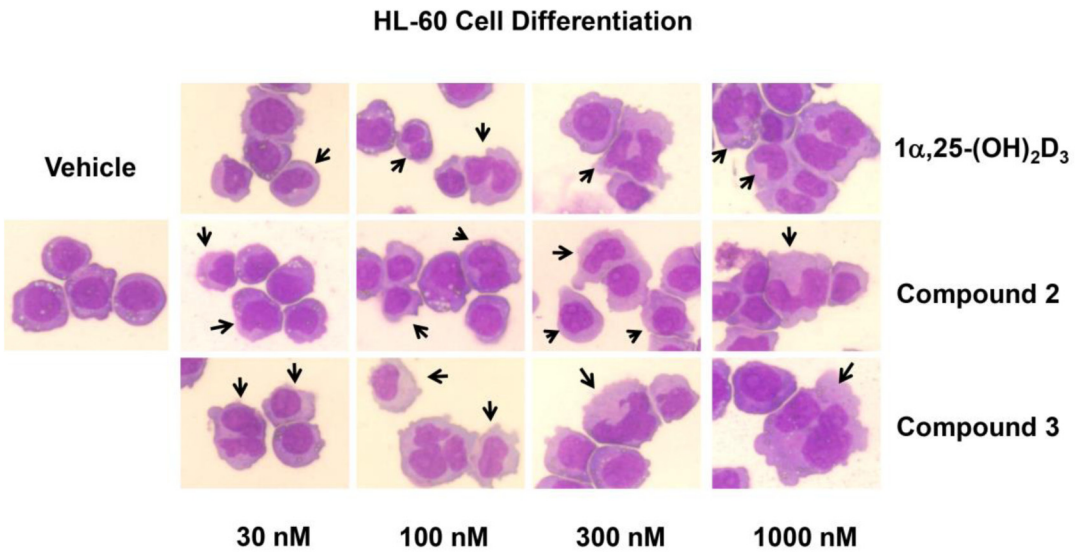


Fig. 4. Induction of HL-60 cell differentiation by analogues **2**, **3** and 1α,25-$(OH)_2$-D_3.

(m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 140.8, 132.9, 130.5, 127.8, 127.4, 124.5, 60.3, 35.3, 33.7, 25.0, 14.3; HRMS (ESI): Calcd for $C_{12}H_{15}BrO_2$ $[M+H]^+$ 271.0334 and 273.0313, found $[M+H]^+$ 271.0342 and 273.0321.

*6.1.4. Ethyl 4-(3-bromophenyl)butanoate (**6b**)*

This compound was prepared from **5b** by the same procedure as described for compound **6a**. Colorless oil; yield: 93%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.33 (d, 2H, J = 9 Hz), 7.15 (t, 1H, J = 7.8 Hz), 7.11 (d, 1H, J = 7.8 Hz), 4.13 (q, 2H, J = 7.2 Hz), 2.62 (t, 2H, J = 7.2 Hz), 2.31 (t, 2H, J = 7.8 Hz), 1.95 (m, 2H), 1.26 (t, 3H, J = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 143.8, 131.5, 130.0, 129.1, 127.2, 122.5, 60.4, 34.8, 33.5, 26.3, 14.3; HRMS (ESI): Calcd for $C_{12}H_{15}BrO_2$ $[M+H]^+$ 271.0334 and 273.0313, found $[M+H]^+$ 271.0338 and 273.0317.

*6.1.5. 1-Bromo-2-(4-(methoxymethoxy)-4-methylpentyl)benzene (**7a**)*

To a solution of **6a** (8.0 g, 29.5 mmol) in THF (60 mL) at −10 °C was slowly added methylmagnesium bromide (24.6 mL, 3.0 M in THF, 73.8 mmol). The mixture was allowed to warm to room temperature and stirred for 8 h. The reaction mixture was quenched with aqueous NH_4Cl, extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The obtained crude tertiary alcohol was dissolved in 70 mL CH_2Cl_2, followed by addition of diisopropylethylamine (14.9 mL, 90.1 mmol) and MOMCl (6.5 mL, 84.6 mmol). After stirring at room temperature for 3 h, the reaction mixture was extracted with CH_2Cl_2, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica (hexane/AcOEt = 50:1) to give **7a** (7.15 g, 94%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.52 (d, 1H, J = 7.8 Hz), 7.22 (d, 2H, J = 4.2 Hz), 7.03–7.06 (m, 1H), 4.69 (s, 2H), 3.36 (s, 3H), 2.72 (t, 2H, J = 7.8 Hz), 1.66–1.71 (m, 2H), 1.58–1.61 (m, 2H), 1.21 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 141.8, 132.8, 130.3, 127.5, 127.4, 124.5, 91.0, 76.2, 55.2, 41.4, 36.6, 26.4, 26.3, 24.5; HRMS (ESI): Calcd for $C_{14}H_{21}BrO_2$ $[M+Na]^+$ 323.0623 and 325.0602, found $[M+Na]^+$ 323.0625 and 325.0604.

*6.1.6. 1-Bromo-3-(4-(methoxymethoxy)-4-methylpentyl)benzene (**7b**)*

This compound was prepared from **6b** by the same procedure as described for compound **7a**. Colorless oil; yield: 92%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34 (s, 1H), 7.32 (d, 1H, J = 6 Hz), 7.14 (t, 1H, J = 6 Hz), 7.11 (d, 1H, J = 6 Hz), 4.68 (s, 2H), 3.36 (s, 3H), 2.57 (t, 2H, J = 8 Hz), 1.64–1.70 (m, 2H), 1.51–1.54 (m, 2H), 1.21 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 144.9, 131.5, 129.9, 128.9, 127.1, 122.4, 91.0, 76.1, 55.2, 41.5, 36.0, 26.3, 26.3, 25.8; HRMS (ESI): Calcd for $C_{14}H_{21}BrO_2$ $[M+Na]^+$ 323.0623 and 325.0602, found $[M+Na]^+$ 323.0631 and 325.0610.

*6.1.7. (1α,3β)-1,3-Bis(tert-butyldimethylsilyloxy)androsta-5,7-dien-17-hydrazone (**10**)*

To a solution of ketone **9** (0.56 g, 1.05 mmol) in ethanol (5 mL) was added hydrazine hydrate (0.22 mL, 4.51 mmol) and catalytic hydrazine sulfate (1 mg) in water (0.10 mL). After stirring at room temperature for 18 h, the mixture was poured into cold water, and the precipitate was collected to give hydrazone **10** (0.50 g, 87%) as a white solid. Mp: 147–150 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.60 (d, 1H, J = 4.8 Hz), 5.41 (d, 1H, J = 4.8 Hz), 4.79 (brs, 2H), 3.97–4.07 (m, 1H), 3.71 (s, 1H), 2.85 (t, 1H, J = 6 Hz), 2.25–2.40 (m, 5H), 1.90–2.05 (m, 4H), 1.52–1.75 (m, 6H), 1.38–1.46 (m, 1H), 0.93 (s, 3H), 0.88 (s, 9H), 0.87 (s, 9H), 0.81 (s, 3H), 0.10 (s, 3H), 0.06 (s, 3H), 0.05 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.6, 138.5, 138.3, 119.8, 115.4, 73.9, 66.3, 52.1, 44.3, 42.9, 40.9, 39.0, 38.0, 33.9, 26.0, 25.9, 24.2, 22.3, 20.4, 18.2, 18.0, 17.0, 16.9, -3.8, -4.3, -4.5, -5.0; HRMS (ESI): Calcd for $C_{31}H_{56}N_2O_2Si_2$ $[M+H]^+$ 545.3959, found $[M+H]^+$ 545.3962.

*6.1.8. (1α,3β)-1,3-Bis(tert-butyldimethylsilyloxy)-17-iodoandrosta-5,7,16-triene (**11**)*

To a stirred solution of iodine (0.50 g, 1.97 mmol) in anhydrous THF (14 mL) and Et_2O (7 mL) at 0 °C was added 1,1,3,3-tetramethylguanidine (TMG, 0.65 mL, 5.18 mmol). A THF solution (8 mL) of **10** (0.50 g, 0.92 mmol) was added dropwise into the iodine solution over 2 h to maintain the reaction temperature at 0 °C. After stirring for 1 h, saturated solution of sodium sulfite was added, and the reaction mixture was extracted with AcOEt, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude iodide **11** (0.59 g) was obtained without further purification.

*6.1.9. (1α,3β)-17-[2-(4-Hydroxy-4-methylpentyl)phenyl]androsta-5,7,16-trien-1,3-diol (**13a**)*

To a stirred solution of compound **7a** (2.07 g, 6.87 mmol) in THF (23 mL) at −78 °C was added *n*-butyllithium (2.3 M solution in

hexanes, 2.50 mL, 5.73 mmol). After stirring for 7 min, zinc chloride (0.5 M solution in THF, 15.7 mL, 7.85 mmol) was added. The mixture was stirred for another 10 min, and then the cooling bath was removed and the stirring was continued for 30 min to form the zinc derivative solution. In parallel, the crude iodide **11** (0.59 g) was dissolved in anhydrous THF (15 mL) and $Pd(PPh_3)_4$ (140 mg) was added. The resulting solution was immediately transferred to the zinc derivative solution via a double-end needle under nitrogen atmosphere. The reaction mixture was stirred for 18 h at room temperature. Aqueous NH_4Cl was added, and the mixture was extracted with AcOEt, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was chromatographed on silica gel (hexane/AcOEt = 50:1) to afford crude **12a** (324 mg).

A solution of crude silyl ether **12a** and TBAF (680 mg, 2.16 mmol) in THF (10 mL) was refluxed for 4 h. The solvent was evaporated, and the residue was extracted with AcOEt, washed with water, dried over Na_2SO_4, filtered, and concentrated. The residue was dissolved in MeOH (7 mL) and TsOH (157 mg, 0.91 mmol) was added. The reaction mixture was stirred at room temperature for 10 min. Et_3N (0.5 mL) was added, and the solvent was evaporated. The residue was chromatographed on silica gel (hexane/AcOEt = 1:3) to afford **13a** (137 mg) as a white solid in 31% yield from compound **10**. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.25 (d, 1H, J = 7.2 Hz), 7.20 (t, 1H, J = 7.2 Hz), 7.13 (t, 1H, J = 7.2 Hz), 7.08 (d, 1H, J = 7.2 Hz), 5.78 (d, 1H, J = 3 Hz), 5.60 (s, 1H), 5.53 (s, 1H), 4.02–4.13 (m, 1H), 3.77 (s, 1H), 2.82–2.87 (m, 1H), 2.55–2.62 (m, 4H), 2.32–2.42 (m, 3H), 2.26–2.29 (m, 1H), 1.73–1.78 (m, 2H), 1.58–1.66 (m, 8H), 1.47–1.54 (m, 2H), 1.20 (s, 6H), 0.96 (s, 3H), 0.98 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.0, 145.5, 144.5, 142.9, 140.7, 140.3, 136.4, 128.8, 127.8, 124.9, 122.0, 115.0, 72.6, 71.0, 65.4, 56.0, 50.0, 44.0, 42.5, 40.2, 34.5, 33.8, 30.8, 30.6, 29.8, 29.3, 26.9, 21.0, 20.9, 17.5, 16.5; HRMS (ESI): Calcd for $C_{31}H_{42}O_3$ $[M+H]^+$, 463.3207, found $[M+H]^+$ 463.3210.

6.1.10. (1α,3β)-17-[3-(4-Hydroxy-4-methylpentyl)phenyl]androsta-5,7,16-trien-1,3-diol (**13b**)

This compound was prepared from **10** by the same procedure as described for compound **13a** in 30% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.19–7.24 (m, 3H), 7.07 (d, 1H, J = 7.2 Hz), 5.94 (t, 1H, J = 2.4 Hz), 5.78 (dt, 1H, J = 6.0, 2.4 Hz), 5.53 (dt, 1H, J = 6.0, 2.4 Hz), 4.09 (ddd, 1H, J = 4.8, 6.0, 10.2 Hz), 3.81 (s, 1H), 2.82 (t, 1H, J = 8.4 Hz), 2.62 (t, 2H, J = 7.8 Hz), 2.55–2.59 (m, 1H), 2.52 (t, 1H, J = 7.2 Hz), 2.34–2.40 (m, 2H), 2.14–2.18 (m, 2H), 1.88 (ddd, 1H, J = 5.4, 12.0, 17.4 Hz), 1.78 (dt, 1H, J = 1.8, 13.2 Hz), 1.67–1.73 (m, 3H), 1.51–1.59 (m, 7H), 1.21 (s, 6H), 1.08 (s, 3H), 1.02 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.6, 142.2, 140.0, 137.2, 136.4, 128.1, 127.0, 126.7, 126.6, 124.1, 122.0, 115.1, 72.7, 71.0, 65.4, 56.3, 47.8, 43.6, 42.5, 40.1, 38.6, 37.6, 36.4, 35.0, 30.5, 29.3, 26.2, 21.1, 21.0, 17.5, 16.2; HRMS (ESI): Calcd for $C_{31}H_{42}O_3$ $[M+H]^+$ 463.3207, found $[M+H]^+$ 463.3208.

6.1.11. (1α,3β)-17-[2-(4-Hydroxy-4-methylpentyl)phenyl]-9,10-secoandrosta-5,7,10(19),16-tetraen-1,3-diol (**2**)

The solution of provitamin D **13a** (60.0 mg, 0.13 mmol) in THF (150 mL) was bubbled with nitrogen at 0 °C for 10 min and then irradiated with a 500 W high pressure mercury lamp until most of the starting material was consumed. The resulting solution was then refluxed for 2 h under the atmosphere of nitrogen. After the removal of the solvent, the residue was purified by column chromatography on silica gel (hexane/AcOEt = 1:3) followed by a reversed-phase HPLC [20 mm × 25 cm YMC-Pack ODS-AQ column, 8 mL/min, MeOH/H_2O (80:20)] to give the pure compound **2** (10 mg, 16%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.24 (d, 1H, J = 7.8 Hz), 7.19 (dt, 1H, J = 1.2, 7.8 Hz), 7.12 (dt, 1H, J = 1.2, 7.8 Hz), 7.08 (d, 1H, J = 7.8 Hz), 6.40 (d, 1H, J = 10.8 Hz), 6.18 (d, 1H, J = 10.8 Hz), 5.56 (s, 1H), 5.37 (s, 1H), 5.06 (s, 1H), 4.48 (t, 1H, J = 9.6 Hz), 4.25 (t, 1H, J = 9.6 Hz), 2.84 (dd, 1H, J = 3.6, 13.2 Hz), 2.60–2.65 (m, 4H), 2.45 (dt, 1H, J = 2.4, 11.4 Hz), 2.35 (dd, 1H, J = 6.6, 13.2 Hz), 2.21–2.33 (m, 1H), 2.03–2.08 (m, 1H), 1.91–1.96 (m, 1H), 1.81–1.87 (m, 1H), 1.70–1.76 (m, 1H), 1.44–1.68 (m, 8H), 1.21 (s, 6H), 0.86 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 153.2, 147.7, 142.2, 140.8, 137.0, 133.3, 128.9, 128.8, 127.2, 126.8, 124.9, 124.9, 117.2, 111.8, 71.0, 70.8, 66.9, 58.3, 52.2, 45.2, 43.9, 42.88, 35.1, 33.8, 30.4, 29.3, 29.2, 28.6, 27.0, 23.4, 17.1; HRMS (ESI): Calcd for $C_{31}H_{42}O_3$ $[M+H]^+$, 463.3207, found $[M+H]^+$ 463.3218; UV (EtOH) λ_{max} 262 nm (ε = 17860).

6.1.12. (1α,3β)-17-[3-(4-Hydroxy-4-methylpentyl)phenyl]-9,10-secoandrosta-5,7,10(19),16-tetraen-1,3-diol (**3**)

this compound was prepared from **13b** by the same procedure as described for compound **2** in 14% yield. H NMR (600 MHz, $CDCl_3$) δ 7.18–7.23 (m, 3H), 7.06 (d, 1H, J = 7.2 Hz), 6.40 (d, 1H, J = 11.4 Hz), 6.17 (d, 1H, J = 11.4 Hz), 5.89 (s, 1H), 5.36 (s, 1H), 5.05 (s, 1H), 4.47 (brs, 1H), 4.26 (brs, 1H), 2.84 (dd, 1H, J = 4.2, 12.6 Hz), 2.58–2.63 (m, 4H), 2.33–2.42 (m, 2H), 2.14–2.18 (m, 1H), 2.04–2.07 (m, 2H), 1.91–1.95 (m, 1H), 1.80–1.86 (m, 2H), 1.67–1.76 (m, 3H), 1.51–1.58 (m, 6H), 1.21 (s, 6H), 0.97 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.5, 147.7, 142.2, 142.1, 137.2, 133.3, 128.1, 126.9, 126.8, 126.3, 124.9, 124.1, 117.4, 111.8, 71.0, 70.8, 66.9, 58.6, 50.0, 45.2, 43.6, 42.9, 36.4, 35.5, 30.0, 29.3, 29.2, 28.6, 26.3, 23.6, 17.5; HRMS (ESI): Calcd for $C_{31}H_{42}O_3$ $[M+NH_4]^+$ 480.3466, found 480.3472; UV (EtOH) λ_{max} 260 nm (ε = 17880).

6.2. VDR binding assay

The bovine thymus VDR receptor was obtained from Yamasa Biochemical (Chiba, Japan) and dissolved in 0.05 M phosphate buffer (pH 7.4) containing 0.3 M KCl and 5 mM dithiothreitol immediately prior to use. The receptor solution (500 mL) was premixed with 50 mL of an ethanol solution of 1α,25-dihydroxyvitamin D_3 or an analogue at various concentrations for 60 min at 25 °C, prior to the before addition of [^{3}H]-1α,25-dihydroxyvitamin D_3 (50 mL). The receptor mixture was then left to stand overnight with 0.1 nM [^{3}H]-1α,25-dihydroxyvitamin D_3 at 4 °C. The bound and free [^{3}H]-1α,25-dihydroxyvitamin D_3 were separated by treatment with a dextran-coated charcoal (Norit SX-II) suspension (200 mL) for 30 min at 4 °C, followed by centrifugation at 3000 rpm for 10 min. The supernatant (500 mL) was mixed with Insta-Gel® Plus (9.5 mL) (PerkinElmer, USA) and the radioactivity was counted. The relative potency of the analogues was calculated from the concentration required to displace 50% of the [^{3}H]-1α,25-dihydroxyvitamin D_3 from the receptor compared with the activity of 1α,25-dihydroxyvitamin D_3, which was assigned as 100 by definition.

6.3. Cell proliferation assay

Human breast cancer MCF-7 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Invitrogen, CA), penicillin (100 units/mL) and streptomycin (100 μg/mL), and were grown at 37 °C and 5% CO_2 conditions.

MCF-7 cell was plated into 96-well plates at a density of 4×10^3 cells/well and treated with compound **2**, **3** or calcitriol (**1**) at indicated concentrations for 72 h, followed by MTT assay as described previously [34].

6.4. HL-60 cell differentiation assay

Human acute promyelocytic leukemia cell line HL-60 was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Invitrogen, CA), penicillin (100 units/mL) and streptomycin (100 μg/mL), and were grown at 37 °C and 5% CO_2 conditions.

HL-60 Cells were treated with compound **2**, **3**, calcitriol (**1**) for 48 h at 30–1000 nM. After the treatment, cells were transferred to slides with cytospin and applied for Wright's–Giemsa staining. Forty microscopic view fields were analyzed and photographed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.08.031.

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