Catalytic asymmetric syntheses and biological activities of singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D_3 A-ring analogs in cancer cell differentiation and apoptosis

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Background: 1α,25-Dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) has been shown to modulate not only proliferation and differentiation, but also apoptosis in malignant cells, indicating that it could be useful for treating cancer. Little information is available concerning the structural motifs of the 1α,25$(OH)_2D_3$ molecule responsible for modulation of differentiation and apoptosis, however. We set out to synthesize singly dehydroxylated A-ring analogs of 19-nor-1α,25$(OH)_2D_3$ in a catalytic asymmetric fashion, and to investigate their biological activities in leukemia HL-60 cells.

Results: A series of singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D_3 A-ring analogs were synthesized using a combinatiorial sequence of regioselective propiolate-ene reaction and catalytic asymmetric carbonyl-ene cyclization. Surprisingly, the analogs could be clearly divided into two categories; one group, bearing 1α-hydroxy or 3β-hydroxy groups in the A-ring, were potent differentiators and the second group, bearing 1β-hydroxy or 3α-hydroxy groups, were potent stimulators of apoptosis.

Conclusions: We have clearly identified the structural motifs of 19-nor-1α,25$(OH)_2D_3$ analogs responsible for differentiation and apoptosis in HL-60 cells. These findings will provide useful information not only for development of therapeutic agents for treatment of leukemia and other cancers, but also for structure–function studies of 1α,25$(OH)_2D_3$.

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Introduction

The hormonally active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) [1], has been recognized as a potent regulator of calcium homeostasis and bone growth. It is also a potent differentiator of immature hematopoietic cells [2] and a potent growth inhibitor of various types of cancer cells, including breast [3], prostate [4] and colon [5], although the sensitivity of prostate cancer cells to the antiproliferative effects of 1α,25$(OH)_2D_3$ varies [6]. These observations have suggested a potential therapeutic application of this hormone in cancer, acute promyelocytic leukemia and psoriasis [7–9]. The mechanisms of growth inhibition and cell differentiation activities and the basis for the variability in antiproliferative effects of 1α,25$(OH)_2D_3$ on certain malignant cells, such as breast [10] and prostate cancer cells [11], is not understood, however. Biological activities of 1α,25$(OH)_2D_3$ are predominantly expressed through a transcriptional regulation resulting from the interaction of 1α,25$(OH)_2D_3$ with the vitamin D receptor (VDR) [12], which is a member of the superfamily of nuclear receptors [13], similar to the thyroid hormone receptor [14], retinoic acid receptor [15], estrogen receptor [16] and glucocorticoid receptor [17]. The 1α,25$(OH)_2D_3$-liganded VDR forms a heterodimer with the retinoid X receptor (RXR) [18,19], which binds to specific DNA sequences, vitamin D-responsive elements (VDREs), to induce transcription [20,21]. An ideal analog of 1α,25$(OH)_2D_3$ would, therefore, possess a high binding affinity for VDR, be able to form a stable VDR–RXR complex and bind strongly to VDREs. From extensive structure–function studies of the vitamin D molecule, it has been assumed that both the hydroxy groups at C-1 and C-25, and possibly the hydroxyl group at C-3, are essential for binding to VDR, which in turn results in expression of the hormonal activities [22]. However, Boehm *et al.* [23] recently reported that a nonsecosteroidal vitamin D analog, 2′-[4-(2-hydroxy-3,3-dimethylbutoxy)-3-methylphenyl]-2′-[4-(2,3-dihydroxypropoxy)-3-methyphenyl]pentane (LG190178), exerts VDR-modulating activities at the molecular level, as well as *in vivo*. DeLuca and coworkers [24] have reported that 19-nor analogs of 1α,25$(OH)_2D_3$, in which an exocyclic methylene group at C-10 on the A-ring is replaced by two hydrogen atoms, have selective activity profiles that combine a high potency for inducing differentiation of malignant cells with very low or nonexistent bone calcitropic activity. Furthermore, 1α,2α,25-trihydroxy-19-nor-vitamin

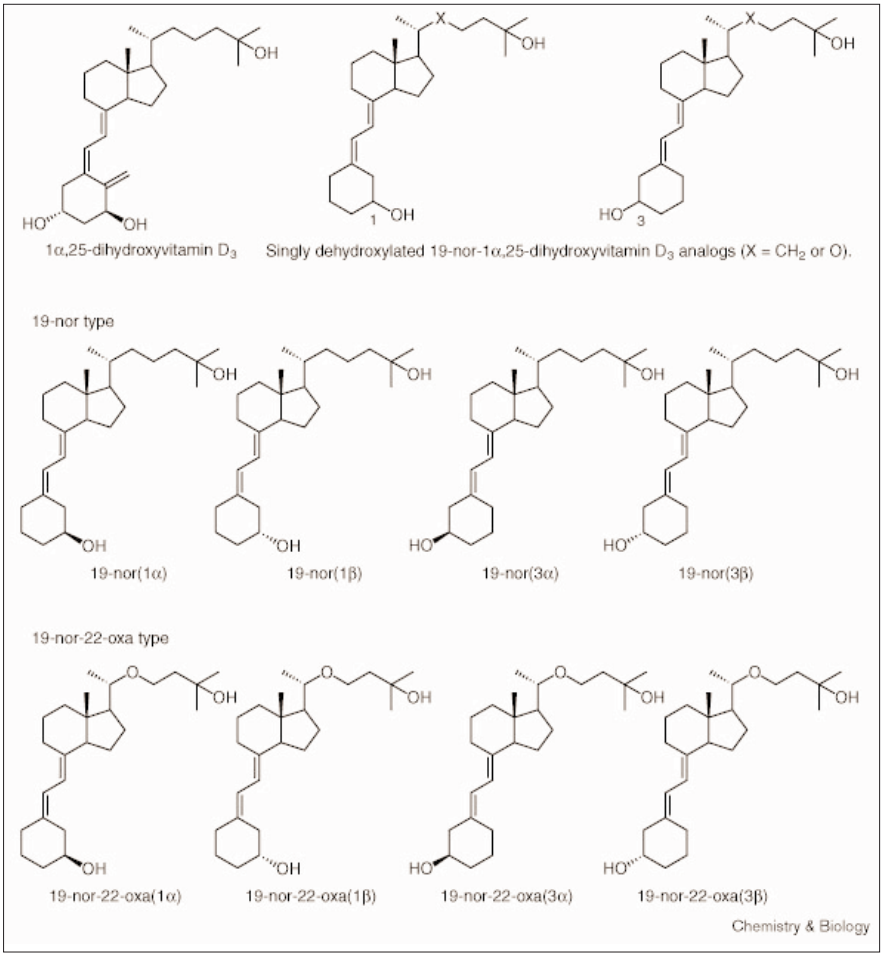
D_3, 1α,2β,25-trihydroxy-19-nor-vitamin D_3 and their 2-alkoxy analogs have been shown to exhibit selective activity in stimulating intestinal calcium transport while having little or no involvement in mobilizing bone calcium [25]. The elucidation of the possible roles of the 1α-hydroxy group, 3β-hydroxy group, and an exocyclic methylene group at C-10 on the A-ring of 1α,25$(OH)_2D_3$ might, therefore, lead to the development of new drugs that are useful for treating cancer and immune diseases. We previously evaluated the biological activities of both series of 19-nor-22-oxa-1α,25$(OH)_2D_3$ and singly dehydroxylated A-ring analogs of 19-nor-1α,25$(OH)_2D_3$ with respect to binding affinities for VDR and vitamin D binding protein (DBP) and transactivation on a rat 25-hydroxyvitamin D_3-24-hydroxylase gene promotor, which has two VDREs, in transfected rat osteosarcoma ROS 17/2.8 cells [26,27]. Surprisingly, several 19-nor analogs were found to be transcriptionally slightly less active than 1α,25$(OH)_2D_3$ or almost comparable to 1α,25$(OH)_2D_3$ despite extremely low binding affinities for VDR. To examine further the biological activity of the 19-nor analogs, we have now investigated their effects on target gene expression, cell-cycle distribution, differentiation into monocytes/ macrophages and apoptosis in human promyelocytic leukemic HL-60 cells. The results revealed an inverse relationship between differentiation-inducing activity and apoptosis-stimulating activity of the 19-nor analogs. One series of 19-nor analogs with 1α- or 3β-hydroxy groups in the A-ring induced cell differentiation and promoted cell-cycle arrest in the G0/G1 phase, but did not stimulate apoptosis. In contrast, the remaining series of 19-nor analogs with 1β-or 3α-hydroxy groups in the A-ring stimulated apoptosis, but were inactive in the regulation of cell differentiation and proliferation. The present findings suggest interesting potential roles for the 1α- and 3β-hydroxy groups or 1β- and 3α-hydroxy groups in the A-ring in regulating cancer cell differentiation or apoptosis, respectively.

Results and discussion

Catalytic asymmetric syntheses of a series of singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D_3 A-ring analogs

The syntheses of both series of singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D_3 A-ring analogs (Figure 1) were established on the basis of our asymmetric

Figure 1



Chemical structures and names assigned to the singly dehydroxylated A-ring analogs of 19-nor-1α,25$(OH)_2D_3$ and 19-nor-22-oxa-(1α),25$(OH)_2D_3$.

catalytic carbonyl-ene cyclization [28,29] using a binaphthol-derived titanium (BINOL-Ti) complex (**1**; Figure 2) [30–32]. The key feature in the syntheses of the A-ring analogs is the stereospecific transformation of the (*E*)- and (*Z*)-ene cyclization products (**3**) into the 3-hydroxy and 1-hydroxy A-ring analogs, respectively.

The preparation of the ene-cyclization substrates (**2**) is worth mentioning (Figure 3). The ene-cyclization substrate **2** was prepared through the reaction sequence starting from regioselective propiolate-ene reaction of homoallylic ether (**4**) [33] with methyl propiolate (**5**) using $EtAlCl_2$ as the initiator [34]. The regioselectivities of the propiolate-ene reaction, namely H- versus H′-shifts leading to the newly formed olefins **6** and **7**, respectively, critically depend on the steric and electronic effects of the alcohol-protecting groups (P) employed. It should be noted here that the sterically highly demanding triisopropylsilyl (TIPS) group gave higher (94%) regioselectivity than those obtained with benzyl (Bn) and *tert*-butyldiphenylsilyl (TBDPS) groups. Reduction of the α,β-unsaturated ester **6**, first with magnesium in methanol and then with lithium aluminum hydride, gave alcohol **8**. The Swern oxidation of alcohol **8** provided the ene-cyclization substrate, aldehyde **2**.

The key step of the syntheses of singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D_3 A-ring analogs was carried out by tuning the BINOL ligands for the catalytic asymmetric syntheses. The geometrical (*Z*/*E*) and enantioselectivities over the newly formed olefin and hydroxy groups, respectively, depend critically on the combination of the BINOL ligands, alcohol-protecting groups and solvents employed (Table 1). It is notable that geminal di-alkyl substituents generally required in the usual catalytic ene cyclization [28,29] are not necessary in the present BINOL-Ti-catalyzed asymmetric carbonyl-ene cyclization.

The characteristics of the catalytic asymmetric carbonyl-ene cyclization in terms of the geometrical and enantioselectivities are shown in Table 1. The carbonyl-ene cyclization of the TBDPS-protected substrate **2a** by the parent (*R*)-BINOL-Ti catalyst (5 mol%) in toluene gave the (*Z*)-ene cyclization product [(*Z*)-**3**)] as the major product in high enantioselectivity for (*S*)-1-hydroxy group (*S*:*R* = 95:5; run 1). Even in toluene, the use of the TIPS-protected substrate **2b** provided, in contrast, the (*E*)-olefinic isomer (runs 3 and 4). These results suggest that not only steric but electronic effects of the alcohol-protecting groups are the determining factors in olefinic selectivity, in other words, in transition-state conformations for ene-cyclizations. In the case of the Bn-protecting group, the (*E*)-olefinic product, (*E*)-**3c**, was obtained as the major product (runs 5–8). Of importance is that the use of sterically bulky and electronically withdrawing 6,6′-dibromo-1,1′-bi-2-naphthol (6-Br-BINOL) ligand [35,36] led to the increase in (*E*)-geometrical selectivity, particularly in dichloromethane, along with the high level of enantioselectivity (*S*:*R* = ~95:5).

Figure 2

Stereospecific transformation of ene products (**3**) obtained via (*R*)-(6-Br)-BINOL-Ti (**1**)-catalyzed asymmetric carbonyl-ene cyclization to singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D_3 analogs.

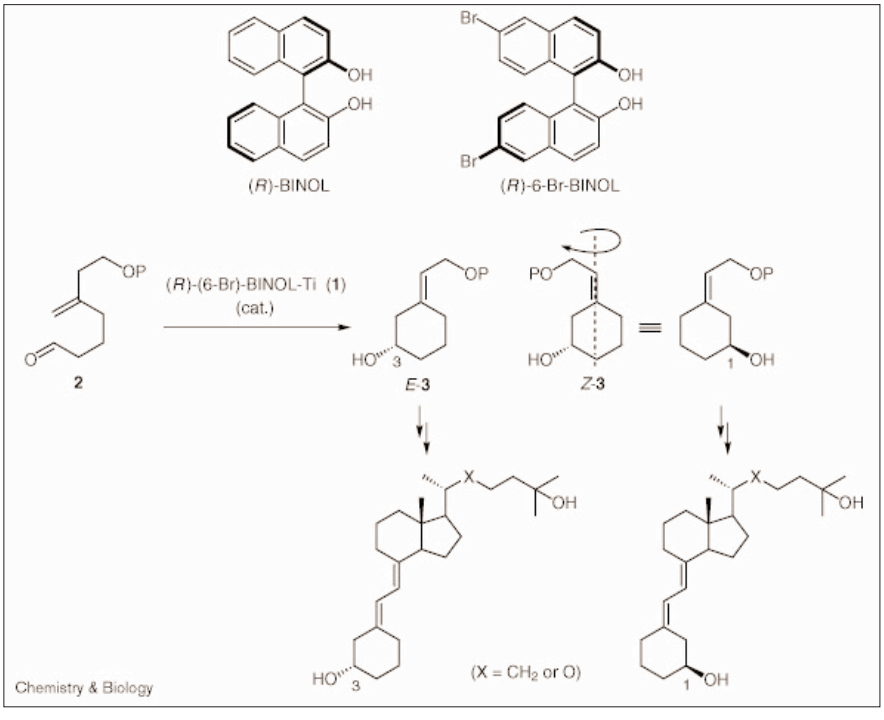
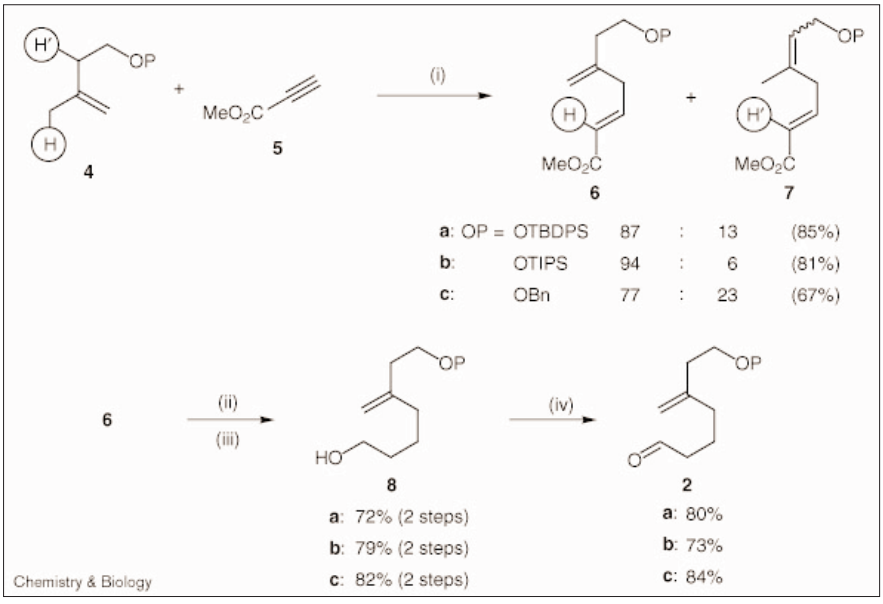


Figure 3



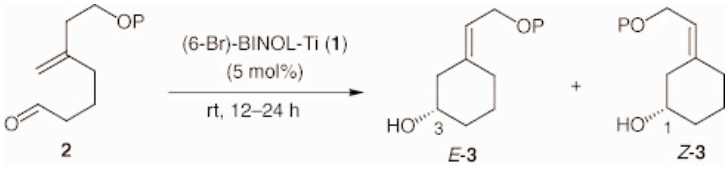
Preparation of ene substrates (**2**) through regioselective propiolate-ene reaction. Reagents and conditions: (i) $EtAlCl_2$, CH_2Cl_2, rt; (ii) Mg, MeOH, rt; (iii) $LiAlH_4$, Et_2O, 0°C; (iv) dimethyl sulfoxide (DMSO), $(COCl)_2$, NEt_3, CH_2Cl_2, −78°C. **a**, **b** and **c** represent the protecting groups TBDPS, TIPS and Bn, respectively.

The enantiomeric excess of the products was determined by chiral high performance liquid chormatography (HPLC; DAICEL CHIRALPAK AD, eluent, hexane/propan-2-ol = 10:1, flow rate 0.5 ml/min, detection 254 nm) and chiral capillary gas chromatography (CP-cyclodextrin-B-2,3,6-M-19, 0.32 mm i.d. × 25 m, carrier gas, N_2 (1.0 ml/min); column temp. 155°C; injection temp. 200°C) analyses and/or by the Mosher method. The absolute configuration of the products was determined by the Mosher method after transformation to the corresponding (*S*)- and (*R*)-α-methoxy-α-trifluoromethyl phenylacetyl (MTPA) esters. The absolute configuration of the newly created stereogenic center at C-1 or C-3 is primarily controlled by the chirality of the (6-Br)-BINOL-Ti catalysts (**1**): the (*R*)-(6-Br)-BINOL-Ti catalysts (**1**) lead to the (*S*)-1- or 3-hydroxy products. The employment of the enantiomeric catalysts, (*S*)-(6-Br)-BINOL-Ti complexes (**1**), provides the opposite enantiomers, (1*R*)- and (3*R*)-hydroxy A-ring isomers.

The (*E*/*Z*)-stereoselectivity of 6-(2,4) [29] carbonyl-ene cyclization of **2** catalyzed by a (6-Br)-BINOL-Ti complex (**1**) depends on the allylic (1,2) strain (T_E) and steric repulsion with the carbonyl/Lewis acid complex (T_Z; Figure 4); in the ene cyclizations, the carbon numbers where the tether connects the [1,5]-hydrogen shift system are indicated as (m,n). A numerical prefix, for example 6-(2,4),

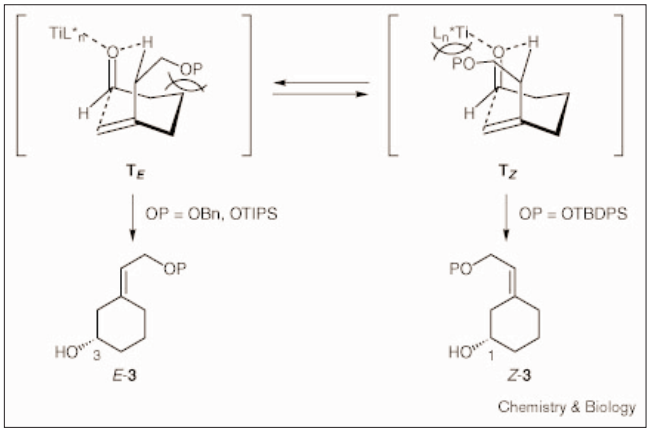
Table 1

Asymmetric carbonyl-ene cyclization catalyzed by (6-Br)-BINOL-Ti complexes (1).



Run	1	2 (OP)	Solvent	Yield	*E*-3 (% ee):*Z*-3 (% ee)
1	(*R*)-BINOL-Ti	**2a** (OTBDPS)	Toluene	88%	37 (76% ee):63 (90% ee)
2	(*R*)-BINOL-Ti	**2a** (OTBDPS)	CH_2Cl_2	92%	47 (78% ee):53 (84% ee)
3	(*R*)-BINOL-Ti	**2b** (OTIPS)	Toluene	71%	59 (77% ee):41 (nd)
4	(*R*)-(6-Br)-BINOL-Ti	**2b** (OTIPS)	Toluene	68%	62 (85% ee):38 (nd)
5	(*R*)-BINOL-Ti	**2c** (OBn)	Toluene	55%	61 (85% ee):39 (82% ee)
6	(*R*)-BINOL-Ti	**2c** (OBn)	CH_2Cl_2	53%	64 (88% ee):36 (85% ee)
7	(*R*)-(6-Br)-BINOL-Ti	**2c** (OBn)	Toluene	38%	71 (89% ee):29 (90% ee)
8	(*R*)-(6-Br)-BINOL-Ti	**2c** (OBn)	CH_2Cl_2	51%	77 (88% ee):23 (86% ee)

Figure 4



Transition states of asymmetric carbonyl-ene cyclization catalyzed by (6-Br)-BINOL-Ti complexes.

stands for a (2,4) ene cyclization to give a six-membered ring. The (*E*)- and (*Z*)-alkylidene cyclohexanol products can be easily separated, however. The carbonyl-ene cyclization catalyzed by (*R*)-BINOL-Ti complexes would proceed via chair-like transition states [37]. The carbonyl-ene cyclization affords the (*E*)- and (*Z*)-isomers of the ene cyclization product (**3**) through T_Z and T_E transition states, depending on the balance of acyclic allylic strain with the sterically demanding alcohol-protecting group (T_Z) and repulsion of the bulky (6-Br)-BINOL-Ti catalyst (T_E) with the alkoxymethyl group, respectively. By tuning the electron withdrawing and sterically demanding (6-Br)-BINOL ligand [35,36], (*E*)-geometrical selectivity significantly increases, up to 77%, because of the steric repulsion between the (6-Br)-BINOL-Ti complex and the benzyloxy- or siloxy-methyl moieties.

Although (*Z*)-**3** and its geometrical isomer (*E*)-**3** can be easily separated from each other, they can be transformed, without separation, to the singly dehydroxylated analogs of 19-nor-1α,25(OH)$_2$D$_3$ (Figure 5), which are also easily separated from each other. This is because the geometric and enantiomeric isomers of the ene products (**3**) can be separated as the diastereomers bearing the chiral steroidal C,D rings at the final stage. On a large scale, the ene-cyclization product (**3**) was silylated with dimethylthexylsilyl (TDS) chloride, without separation, to the (1*S*)- and (3*S*)-hydroxy A-ring intermediates (**9**). Further transformation of the intermediate (**9**) via the chloride (**10**) gave the Horner–Emmons–Wittig reagent (**11**). Lythgoe coupling of the C,D ring (**12**: X = CH_2 or O) with the Wittig reagent (**11**) led (via desilylation) to the singly dehydroxylated analogs of 19-nor-1α,25(OH)$_2$D$_3$ (Figure 1). After chromatographic purification, the diastereomerically pure analogs were isolated: HPLC (Inertsil SIL, eluent, hexane/ethyl acetate = 3:1, flow rate 0.5 ml/min) 19-nor(3α): t_R = 24.99 min; 19-nor(3β): t_R = 27.43 min; 19-nor(1α): t_R = 30.87 min; 19-nor(1β): t_R = 34.55 min, HPLC (Zorbax SIL, eluent, hexane/2-propanol/methanol = 91:7:2, flow rate 0.7 ml/min) 19-nor-22-oxa(3α): t_R = 12.04 min; 19-nor-22-oxa(3β): t_R = 12.17 min, HPLC (Inertsil SIL, eluent, hexane/ethyl acetate = 3:1, flow rate 0.8 ml/min) 19-nor-22-oxa(1α): t_R = 13.21 min; 19-nor-22-oxa(1β): t_R = 14.24 min.

Transcriptional potencies of 19-nor(3β) and 19-nor-22-oxa(1α)

Transcriptional potencies of 10^{-8} M 1α,25(OH)$_2$D$_3$ and 19-nor analogs on HL-60 cells containing the rat 25(OH)D$_3$-24-hydroxylase gene promoter (which has two VDREs) are shown in Figure 6. Of the 19-nor analogs, 19-nor(3β) and 19-nor-22-oxa(1α) exhibited ~50% increased potency compared to 1α,25(OH)$_2$D$_3$, whereas the other 19-nor analogs had virtually no potency. These results were consistent with those obtained using rat osteosarcoma ROS17/2.8 cells [27].

19-nor(3β) and 19-nor-22-oxa(1α) inhibit ^{3}H-thymidine incorporation into HL-60 cells

As shown in Figure 7, treatment of HL-60 cells for 3 days with 10^{-7} M 1α,25(OH)$_2$D$_3$ inhibited ^{3}H-thymidine

Figure 5

Transformation of ene products (**3**) to singly dehydroxylated analogs of 19-nor-1α,25(OH)$_2$D$_3$. Reagents and conditions: (i) dimethylthexylsilyl chloride (TDSCl), imidazole, dimethyl formamide (DMF), rt; (ii) Li, *tert*-BuOH, NH_3, Et_2O, −78°C; (iii) N-chlorosuccinimide (NCS), Me_2S, CH_2Cl_2, −20°C to rt; (iv) PhPLi, THF, 0°C; (v) H_2O_2, THF-H_2O, rt; (vi) 12, *n*-BuLi, THF, −78°C; (vii) TBAF, THF, rt.

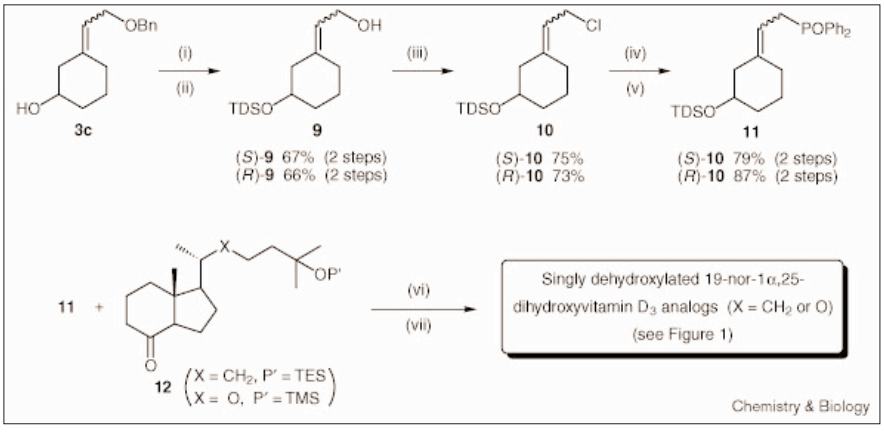
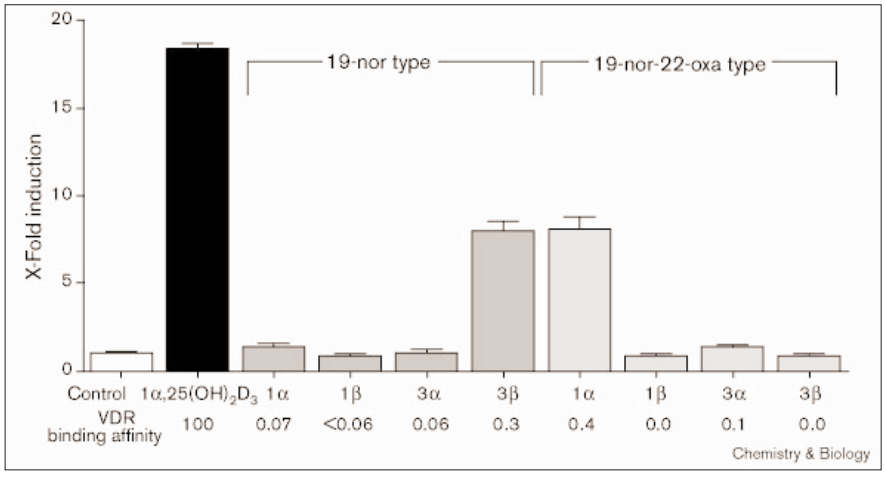


Figure 6



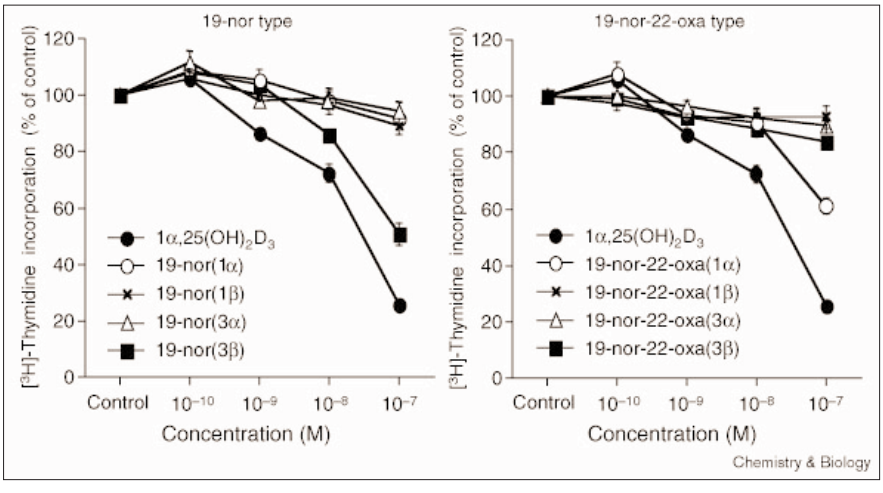
Transcriptional potency of 1α,25(OH)$_2$D$_3$ and the 19-nor-1α,25(OH)$_2$D$_3$ analogs on a rat 25(OH)D$_3$-24-hydroxylase gene promoter in transfected HL-60 cells. The cells were electroplated with a reporter plasmid (pGVB2 vector, Toyo Ink Co., Ltd, Tokyo, Japan) containing the luciferase gene under the control of a rat 25(OH)D$_3$-24-hydroxylase promoter (–291/+9), which has two VDREs, and a pRL-CMV vector as an internal control. After 48 h culture with the compounds, cell lysates were prepared and light production from luciferase was measured as described in the Materials and methods section. Luciferase activities induced by 1α,25(OH)$_2$D$_3$ and its analogs in HL-60 cells were quantified and represented as x-fold induction as compared with luciferase activity observed in the control cells. Results represent the mean of three experiments (values in column) and standard errors (vertical bars) at 10^{-8} M. The VDR binding affinities of all analogs were previously reported [27].

incorporation with an efficacy of ~60%, in agreement with a previous report [27]. Despite extremely low binding affinities for VDR (as reported previously), 10^{-7} M 19-nor(3β) and 19-nor-22-oxa(1α) inhibited ^{3}H-thymidine incorporation with efficacies of ~50% and ~60%, respectively. No other 19-nor analogs inhibited ^{3}H-thymidine incorporation. We previously reported that among the 19-nor analogs tested here, only 19-nor(3β) and 19-nor-22-oxa(1α) bind VDR *in vitro*, with ~0.3% and 0.4% affinities compared with 1α,25(OH)$_2$D$_3$, respectively (Figure 6), whereas they have slightly weaker or comparable potencies for transactivation of the rat 25-hydroxyvitamin D$_3$-24-hydroxylase gene promoter in a co-transfection-luciferase assay. Both 19-nor analogs, therefore, appear to inhibit ^{3}H-thymidine incorporation through a VDR-mediated mechanism in HL-60 cells.

19-nor(3β) and 19-nor-22-oxa(1α) cause HL-60 cells to accumulate in G0/G1

1α,25(OH)$_2$D$_3$ has been shown to promote cell-cycle arrest in the G0/G1 phase in a variety of cell lines including leukemic cells and breast cancer cells [38,39]. To compare the antiproliferative effects of analogs on HL-60 cell growth, we assayed cell-cycle distribution of HL-60 cells treated with 1α,25(OH)$_2$D$_3$ or the 19-nor analogs. Results from three individual experiments after 72 hours by flow cytometry of propidium iodide-stained cells are shown in Table 2. We found that, like 1α,25(OH)$_2$D$_3$, 19-nor(3β) and 19-nor-22-oxa(1α) caused HL-60 cells to accumulate in the G0/G1 phase of the cell cycle. The percentages of HL-60 cells in the S + G2/M phases decreased from 43% (vehicle-treated cells) to 27% by 1α,25(OH)$_2$D$_3$, to 35% by

Figure 7



[^{3}H]-Thymidine incorporation into HL-60 cells treated with either 1α,25(OH)$_2$D$_3$ or the 19-nor-1α,25(OH)$_2$D$_3$ analogs. HL-60 cells were plated at 10^6 cells per well in 24-well plates. 1α,25(OH)$_2$D$_3$ or 19-nor-1α,25(OH)$_2$D$_3$ analogs were added to ethanol vehicle in media at 4 concentrations, from 10^{-7}–10^{-10} M. After 3 days, radioactivity incorporated into the cells was measured by liquid scintillation counter.

Table 2

Cell cycle phase distribution in HL-60 cells treated with 1α,25(OH)$_2$D$_3$ or the analogs.

Compound	G0/G1 (%)	S (%)	G2/M (%)
Control	57.27 ± 1.57	33.48 ± 1.26	9.25 ± 0.33
1α,25(OH)$_2$D$_3$	73.20 ± 1.25**	19.27 ± 1.05***	7.53 ± 0.26*
19-nor(1α)	56.81 ± 1.19**	33.99 ± 1.28***	9.20 ± 0.10*
19-nor(1β)	56.97 ± 1.43**	34.16 ± 1.07***	8.88 ± 0.39*
19-nor(3α)	57.18 ± 1.39**	33.99 ± 0.93***	8.83 ± 0.47*
19-nor(3β)	64.57 ± 0.51*	26.91 ± 1.02*	8.52 ± 0.57*
19-nor-22-oxa(1α)	63.33 ± 0.62*	27.93 ± 0.62*	8.74 ± 0.17
19-nor-22-oxa(1β)	57.92 ± 1.50*	33.58 ± 0.91*	8.50 ± 0.59
19-nor-22-oxa(3α)	59.03 ± 0.83*	31.72 ± 0.71*	9.25 ± 0.29
19-nor-22-oxa(3β)	57.15 ± 1.29*	33.98 ± 1.02*	8.87 ± 0.47

Values sharing the same subscript are significantly different (*< 0.05, **< 0.01, ***< 0.001).

19-nor(3β), and to 37% by 19-nor-22-oxa(1α) at 10^{-7} M. There were no significant differences in cell-cycle distributions of the HL-60 cells treated with the other 19-nor analogs relative to vehicle-treated cells. These results are consistent with those from previous ^{3}H-thymidine incorporation assays. Thus, the HL-60 cell growth inhibition exerted by 1α,25(OH)$_2$D$_3$, 19-nor(3β) and 19-nor-22-oxa(1α) is probably due to inhibition of cellular progression to G0/G1 + S phases via a VDR-mediated mechanism.

19-nor(3β) and 19-nor-22-oxa(1α) cause HL-60 cells to differentiate into monocytes/macrophages

Expression of cell-surface CD11b and CD14 antigens is one of the major differentiation markers of HL-60 cells to monocytes/macrophages. As depicted in Figure 8, 1α,25(OH)$_2$D$_3$, 19-nor(3β) and 19-nor-22-oxa(1α) at 10^{-7} M significantly increased the number of CD11b antigen-expressing cells. CD11b antigen expression increased in both dose- and time-dependent manners in the presence of 1α,25(OH)$_2$D$_3$, 19-nor(3β) or 19-nor-22-oxa(1α) (data not shown). The other 19-nor analogs did not cause HL-60 cells to express CD11b antigen over vehicle-treated cells at any time or dose. 1α,25(OH)$_2$D$_3$ increased the number of CD14 antigen-expressing cells, whereas the 19-nor analogs had weak or virtually no such potency. Regarding morphological changes, the HL-60 cells treated with 1α,25(OH)$_2$D$_3$, 19-nor(3β) or 19-nor-22-oxa(1α) became longer and thinner, giving a macrophage-like appearance. These findings clearly indicate that both 19-nor(3β) and 19-nor-22-oxa(1α) can induce differentiation of HL-60 cells along the macrophage lineage via a VDR-mediated mechanism.

19-nor(1β), 19-nor(3α) and 19-nor-22-oxa(1β) increase the number of apoptotic HL-60 cells

To clarify whether the inhibitory effects of 1α,25(OH)$_2$D$_3$ and 19-nor analogs on HL-60 cell growth correlate with their effects on apoptosis, the number of apoptotic HL-60 cells treated with 1α,25(OH)$_2$D$_3$ or 19-nor analogs was counted and calculated as a percentage of 200 cells scored in each random fluoromicroscopic field of view. Figure 9 shows that 19-nor(1β), 19-nor(3α) and 19-nor-22-oxa(1β), but not 1α,25(OH)$_2$D$_3$ or other 19-nor analogs, induced apoptosis in HL-60 cells. It has been reported that 1α,25(OH)$_2$D$_3$ itself has no apoptosis-stimulating effect on HL-60 cells, although it has an inhibitory effect on drug-induced apoptosis in HL-60 cells [40]. Our findings are consistent with these reports. Surprisingly, 19-nor(1β), 19-nor(3α) and 19-nor-22-oxa(1β), which have unnatural orientations (1 and 3 epimerization, respectively) of the hydroxyl groups at the C-1 and C-3 positions in the A-ring, significantly increased the number of apoptotic HL-60 cells. Moreover, the apoptosis-stimulating potency of

Figure 8

CD11b- and CD14-antigen-positive cell numbers appeared from HL-60 cells treated with either 1α,25(OH)$_2$D$_3$ or the 19-nor-1α,25(OH)$_2$D$_3$ analogs. HL-60 cells were grown and plated at 10^5 cells per well in 24-well plates as described in the Materials and methods section. 1α,25(OH)$_2$D$_3$ or the 19-nor-1α,25(OH)$_2$D$_3$ analogs were added in ethanol vehicle in media at 10^{-7} M and incubation proceeded for 3 days. Cells were harvested and CD11b and CD14 analyses using a fluorescence-activated cell sorter (FACS) were performed.

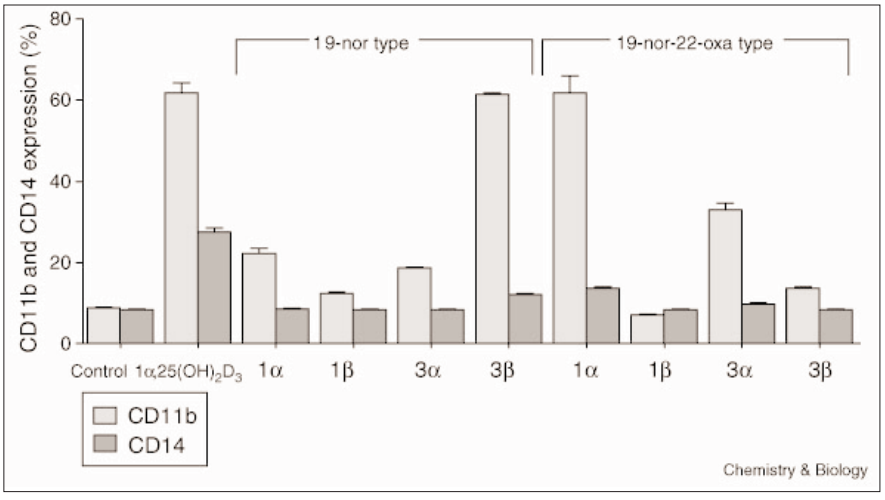
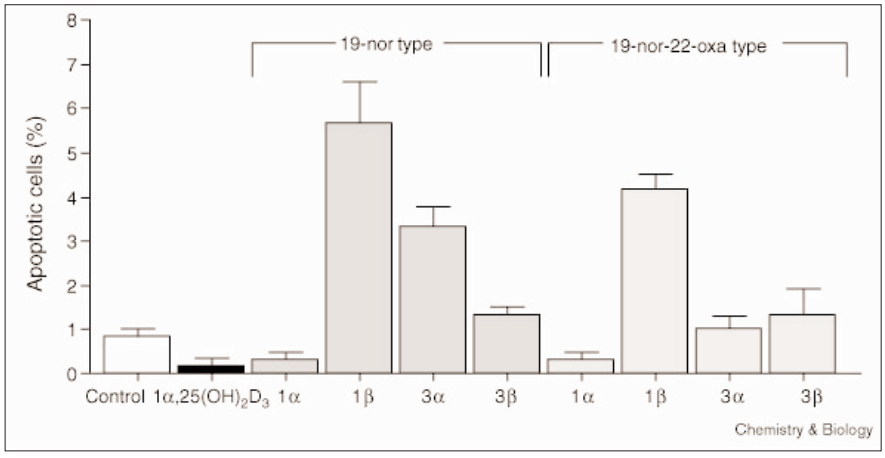


Figure 9



Number of apoptotic cells from HL-60 cells treated with 1α,25(OH)$_2$D$_3$ or the 19-nor-1α,25(OH)$_2$D$_3$ analogs. HL-60 cells were grown and plated at 10^5 cells per well in 24-well plates as described in the Materials and methods section. 1α,25(OH)$_2$D$_3$ or 19-nor-1α,25(OH)$_2$D$_3$ analogs were added to ethanol vehicle in media at 10^{-7} M, and incubation proceeded for 120 h. In duplicate cultures, changes in nuclear morphology that were characteristic of apoptosis were quantified by staining with the DNA-specific fluorochrome bisbenzimide (Hoechst 33258), and data are expressed as percentages of apoptotic cells. Cells with chromatin condensation in the nucleus into three or more fragments were considered to be apoptosed. A minimum of 200 cells in each field were scored. The data represent two independent determinations from three separate experiments.

19-nor(1β) and 19-nor(3α), which bear natural sidechains, appears to be greater than that of 19-nor(1β) and 19-nor(3α), which bear 22-oxa type sidechains. It has been reported that 22-oxa-1α,25(OH)$_2$D$_3$ is more active than 1α,25(OH)$_2$D$_3$ in stimulating monocytic differentiation of HL-60 cells to macrophages through a VDR-mediated mechanism [41]. Our findings coupled with previous findings on 22-oxa-1α,25(OH)$_2$D$_3$ suggest that a 22-oxa-type sidechain functions as an enhancer via a VDR-dependent mechanism, but as a suppressor in a VDR-nondependent mechanism. In addition, it is possible that the orientation of the hydroxyl groups at the C-1 and C-3 positions in the A-ring of 1α,25(OH)$_2$D$_3$ may be structural motifs responsible for controlling two signaling pathways, namely a VDR-dependent mechanism, such as cell differentiation and transactivation of target genes, and a VDR-nondependent mechanism, such as apoptosis.

19-nor(1β), 19-nor(3α) and 19-nor-22-oxa(1β) induce DNA fragmentation in HL-60 cells

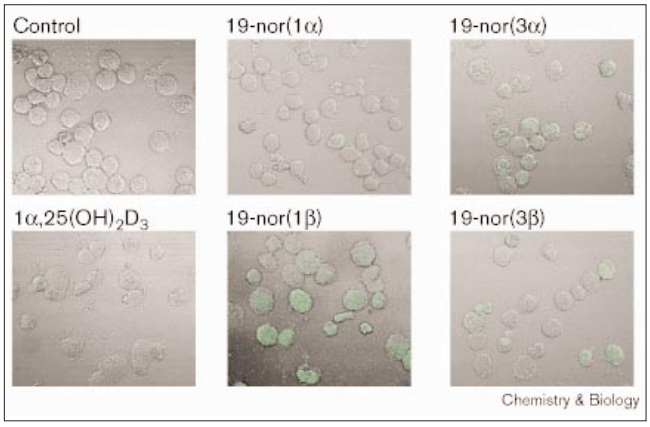
Figures 10 and 11 show fluoromicroscopy images of HL-60 cells treated with 1α,25(OH)$_2$D$_3$ or 19-nor analogs using a Tdt-mediated dUTP nick end-labeling (TUNEL) method. No apoptotic cells were observed in the vehicle- or 1α,25(OH)$_2$D$_3$-treated HL-60 cells. Moreover, no apoptotic cells were observed in the 19-nor analogs bearing 1α- or 3β-hydroxyl groups in the A-ring. In contrast, the 19-nor analogs bearing 1β- or 3α-hydroxyl groups in the A-ring exerted weak, but significant, apoptosis induction.

Figure 12 shows that the 19-nor analogs bearing 1β- or 3α-hydroxyl groups in the A-ring (but not vehicle, 1α,25(OH)$_2$D$_3$ or other 19-nor analogs) caused DNA fragmentation, which is typical of apoptosis. DNA ladders were prominent when cells were treated for 120 h. In addition, fragmentation of DNA by 19-nor(1β) and 19-nor(3α) was more prominent than by 19-nor-22-oxa(1β) and 19-nor-22-oxa(3α), suggesting that the 19-nor analogs that have natural sidechains induce apoptosis more strongly than those with 22-oxa-type sidechains.

We previously reported that two novel 19-nor-1α,25(OH)$_2$D$_3$ analogs, 19-nor(3β) and 19-nor-22-oxa(1α), are comparable to 1α,25(OH)$_2$D$_3$ in stimulating rat 25-hydroxyvitamin D$_3$-24-hydroxylase gene promoter activity in transfected rat osteosarcoma ROS 17/2.8 cells, regardless of their extremely low binding affinities for VDR (0.3% and 0.4%, respectively, compared with that of 1α,25(OH)$_2$D$_3$) [27]. We also found that both analogs have significant antiproliferative activity on HL-60 cells. These results suggested that in 1α,25(OH)$_2$D$_3$ molecules, singly 1α- or 3β-hydroxylated A-rings without the C-19 moiety are key structural motifs for expression of vitamin D action via a VDR-mediated signaling pathway in target cells.

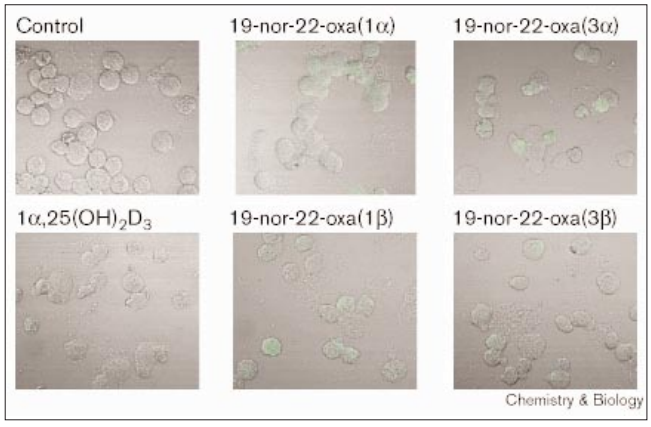
To clarify other possible roles of these hydroxyl groups, we further investigated the biological activities of 19-nor analogs in HL-60 cells with respect to differentiation, cell-cycle distribution and induction of apoptosis. McCarthy *et al.* [42] previously reported that 1α,25(OH)$_2$D$_3$ inhibits proliferation of HL-60 cells and promotes differentiation of HL-60 cells to monocyte/macrophage-like cells without inducing apoptosis. In agreement with this study, we found that treating HL-60 cells with 1α,25(OH)$_2$D$_3$ induced monocytic differentiation and cell-cycle arrest in the G0/G1 phase (Figure 8 and Table 2). Like 1α,25(OH)$_2$D$_3$, 19-nor(3β) and 19-nor-22-oxa(1α) also induced cell-growth arrest and differentiation without

Figure 10



Apoptotic cells from HL-60 cells treated with either vehicle, 1α,25(OH)$_2$D$_3$ or 19-nor-1α,25(OH)$_2$D$_3$ analogs. HL-60 cells were grown and plated at 10^5 cells per well in 24-well plates as described in the Materials and methods section. 1α,25(OH)$_2$D$_3$ or 19-nor-1α,25(OH)$_2$D$_3$ analogs were added to ethanol vehicle in media at 10^{-7} M and incubation proceeded for 120 h. Cells were stained by the TUNEL method and nuclei were visualized by fluorescence microscopy. Green fluorescent-labeled cells are TUNEL positive cells.

Figure 11

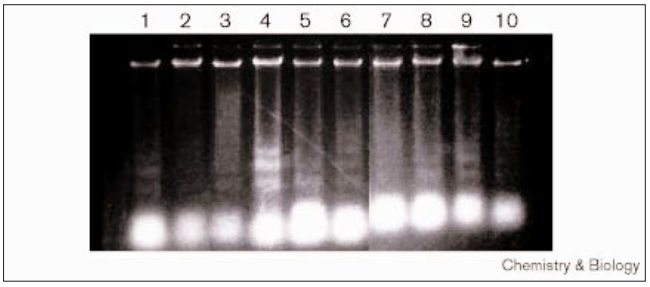


Apoptotic cells from HL-60 cells treated with either vehicle, 1α,25(OH)$_2$D$_3$ or 19-nor-22-oxa-1α,25(OH)$_2$D$_3$ analogs. HL-60 cells were grown and plated at 10^5 cells per well in 24-well plates as described in the Materials and methods section. 1α,25(OH)$_2$D$_3$ or 19-nor-1α,25(OH)$_2$D$_3$ analogs were added to ethanol vehicle in media at 10^{-7} M and incubation proceeded for 120 h. Cells were stained by the TUNEL method and nuclei were visualized by fluorescence microscopy. Green fluorescent-labeled cells are TUNEL positive cells.

inducing apoptosis, although they were much less potent (Figures 8–12 and Table 2). In contrast, 19-nor(1β), 19-nor(3α) and 19-nor-22-oxa(1β), which completely lack VDR binding potency [27] and possess an epimerized hydroxyl group at C-1 or C-3 in the A-ring, all induced apoptosis without inducing cell-growth arrest and differentiation (Figures 8–12 and Table 2). These findings suggest that in 19-nor-1α,25(OH)$_2$D$_3$ analogs both the 1α-hydroxyl and 3β-hydroxyl groups in the A-ring are structural motifs for the induction of monocytic differentiation in HL-60 cells, but not apoptosis, and conversely, both the 1β-hydroxyl and 3α-hydroxyl groups in the A-ring are structural motifs for the induction of apoptosis, but not monocytic differentiation. It is also interesting that an analog with the combination of 22-oxa sidechain and 3α-hydroxyl group appears to have extremely weak VDR-binding affinity and induces monocytic differentiation in HL-60 cells, but not apoptosis. This is the first report demonstrating selective control of apoptosis and monocytic differentiation in HL-60 cells that is dependent on the stereochemistry of hydroxyl groups at the C-1 and C-3 positions in the A-ring of 19-nor-1α,25(OH)$_2$D$_3$ analogs.

A number of vitamin D analogs, including 1α,25(OH)$_2$D$_3$ itself, are known to induce apoptosis in many subtypes of breast cancer and prostate cancer cell lines [43,44]. It has been suggested that 1α,25(OH)$_2$D$_3$ prevents drug-induced apoptosis in HL-60 cells through increased expression of the anti-apoptosis genes Bcl-XL and Mcl-1 [40]. To our knowledge, however, few reports have demonstrated apoptosis induced by 1α,25(OH)$_2$D$_3$ and its active analogs in HL-60 cells [45]. Because HL-60 cells are known to be highly susceptible to 1α,25(OH)$_2$D$_3$ and can be differentiated to monocyte/macrophage-like cells, it is possible that the differentiation-inducing activity of 1α,25(OH)$_2$D$_3$ is much greater than its apoptotic activity, therefore masking its apoptotic activity. In contrast, 1α,25(OH)$_2$D$_3$ is known to be highly anti-proliferative in breast cancer cell line MCF-7 and induces apoptosis via cell-cycle arrest [46]. Our singly dehydroxylated A-ring analogs provided useful probes to assess the biological roles of hydroxyl groups independently and try to resolve some of these conflicting observations. It appears that the stereochemistry of these hydroxyl groups determines whether an analog induces differentiation or apoptosis. To test this hypothesis, further investigations are needed to elucidate the apoptotic effects of 19-nor-1α,25(OH)$_2$D$_3$ analogs on MCF-7 cells. Although the stereochemistry of hydroxyl groups at C-1 and C-3 in the A-ring provides the key structural motifs responsible for both biological activities, it is unclear how the 19-nor-1α,25(OH)$_2$D$_3$ analogs mediate these unique biological responses. It has been reported that 1α,25(OH)$_2$D$_3$ protects HL-60 cells from drug-induced apoptosis [40], and also reduces the apoptotic effects of retinoid N-(4-hydroxyphenyl)-all-*trans*-retinamide [47]. Because antisense inhibition of VDR expression induces apoptosis in U937 cells [48], it has been suggested that the anti-apoptotic effect of 1α,25(OH)$_2$D$_3$ is mediated via binding to VDR. It is likely, therefore, that 19-nor(3β) and 19-nor-22-oxa(1α), which possess VDR binding affinities even at extremely low concentrations, might exert anti-apoptotic effects on HL-60 cells similar to 1α,25(OH)$_2$D$_3$; further investigation is

Figure 12



DNA fragmentation assay of apoptosis in HL-60 cells treated with either vehicle, 1α,25(OH)$_2$D$_3$ or 19-nor-1α,25(OH)$_2$D$_3$ analogs. HL-60 cells were grown and plated at 10^5 cells per well in 24-well plates as described in the Materials and methods section. 1α,25(OH)$_2$D$_3$ or 19-nor-1α,25(OH)$_2$D$_3$ analogs were added to ethanol vehicle in media at 10^{-7} M and incubation proceeded for 120 h. Cells were harvested and fragmentation of chromosomal DNA was assessed by electrophoresis on a 2.0% agarose gel. Lane 1, vehicle (ethanol); lane 2, 1α,25(OH)$_2$D$_3$; lane 3, 19-nor(1α); lane 4, 19-nor(1β); lane 5, 19-nor(3α); lane 6, 19-nor(3β); lane 7, 19-nor-22-oxa(1α); lane 8, 19-nor-22-oxa(1β), lane 9, 19-nor-22-oxa(3α); lane 10, 19-nor-22-oxa(3β).

needed in this matter. In contrast, 19-nor(1β), 19-nor(3α) and 19-nor-22-oxa(1β), which are completely inactive in inducing differentiation and inhibiting proliferation of HL-60 cells due to a lack of VDR binding potency, induce apoptosis in HL-60 cells. Because they do not bind to the VDR, it is clear that their apoptotic effects on HL-60 cells are not mediated by a classical VDR-mediated signalling pathway. At present, we do not have any explanation for the mechanism by which these analogs induce apoptosis in HL-60 cells. Recently, several lines of evidence have been presented concerning the induction of apoptosis in HL-60 cells. It has been suggested that apoptotic agents release cytochrome C, an oxidant, from the mitochondria, which then activates caspase-3 (CPP32), which in turn cleaves the 45 kDa subunit of the DNA fragmentation factor (DFF) to produce an active factor that triggers apoptotic DNA fragmentation [49]. Enigmatically, 1α,25(OH)$_2$D$_3$ appears to increase the mitochondrial anti-apoptotic protein MCL-1 and proliferation-associated protein RAF-1, which in turn reduces the release of mitochondrial cytochrome C and prevents HL-60 cells from undergoing apoptosis [40]. This cytoprotective effect of 1α,25(OH)$_2$D$_3$ appears to be mediated via the binding of 1α,25(OH)$_2$D$_3$ to VDR; it is unlikely, therefore, that 19-nor(1β), 19-nor(3α) and 19-nor-22-oxa(1β) use this signaling pathway. Ceramide, a sphingolipid breakdown product, is known to be a potent inducer of apoptosis in HL-60 cells. It has been shown that 1α,25(OH)$_2$D$_3$ activates sphingosine kinase, which in turn converts ceramide to sphingosin-1-phosphate (SPP) and prevents HL-60 cell ceramide-induced apoptosis [50]. There was a correlation between the time course and dose response of activation of sphingosin kinase by 1α,25(OH)$_2$D$_3$ and protection from apoptosis. This cytoprotective effect of 1α,25(OH)$_2$D$_3$ is also dependent on VDR binding potency. The apoptotic effects of 19-nor analogs may, therefore, occur via this mechanism. In the present study, the apoptosis of HL-60 cells was induced by several 19-nor-1α,25(OH)$_2$D$_3$ analogs, but not by 1α,25(OH)$_2$D$_3$, suggesting that these analogs have a qualitative difference in biological activity compared with 1α,25(OH)$_2$D$_3$. Because these analogs lack the ability to bind VDR, it is reasonable to suggest that these analogs use unknown receptors or recognize unknown enzymes that mediate a novel set of gene expression or metabolism that regulates apoptosis. Further investigation, such as the identification of candidates involved in the induction of apoptosis in HL-60 cells, will help us to understand the biological roles of each hydroxyl group and the exocyclic methylene moiety in the A-ring of 1α,25(OH)$_2$D$_3$.

In summary, we have identified a novel series of 19-nor-1α,25(OH)$_2$D$_3$ analogs with selective control activities for apoptosis and monocytic differentiation of HL-60 cells *in vitro*. These interesting analogs will be studied for their ability to regulate target gene expressions *in vitro* and control myeloid leukemias *in vivo*.

Significance

1α,25-Dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$), the active form of vitamin D$_3$, acts through the vitamin D receptor to elicit a number of activities. Because these activities include differentiation, proliferation and apoptosis in malignant cells, 1α,25(OH)$_2$D$_3$ or its derivatives could be useful for treating cancer. There is very little known about the various structural motifs of 1α,25(OH)$_2$D$_3$ that are responsible for modulating these different activities, however. In order to investigate these motifs, we synthesized a series of singly dehydroxylated 19-nor-1α,25-dihydroxyvitamin D$_3$ A-ring analogs in a catalytic asymmetric fashion and investigated their biological activities in leukemia HL-60 cells. To our surprise, the analogs could be clearly divided into two groups. A-ring analogs that contain 1α-hydroxy or 3β-hydroxy groups induce cellular differentiation and analogs that bear 1β-hydroxy or 3α-hydroxy groups are potent stimulators of apoptosis. These observations should help in the design and development of therapeutic agents for treating leukemia and other cancers.

Materials and methods

Chemicals

1α,25(OH)$_2$D$_3$ was purchased from Solvay-Duphar Co., Weesp, The Netherlands. The 19-nor-1α,25(OH)$_2$D$_3$ analogs included 19-nor-3β-dehydroxy-1α,25(OH)$_2$D$_3$ [19-nor(1α)], 19-nor-3β-dehydroxy-1β,25(OH)$_2$D$_3$ [19-nor(1β)], 19-nor-3-epi-25(OH)D$_3$ [19-nor(3α)], and 19-nor-25(OH)D$_3$ [19-nor(3β)]. The singly dehydroxylated A-ring analogs of 19-nor-22-oxa-1α,25(OH)$_2$D$_3$ included 19-nor-3β-dehydroxy-22-oxa-1α,25(OH)$_2$D$_3$ [19-nor(1α)], 19-nor-3β-dehydroxy-22-oxa-1β,25-(OH)2D$_3$ [19-nor(1β)], 19-nor-3-epi-22-oxa-25(OH)D$_3$ [19-nor(3α)], and 19-nor-22-oxa-25(OH)D$_3$ [19-nor(3β)]. All analogs were dissolved in aldehyde-free absolute ethanol as stock solutions at 10^{-4} M and stored at −35°C protected from light.

Transcriptional activity on a rat 25(OH)D$_3$-24-hydroxylase gene promoter in transfected HL-60 cells

Human promyelocytic leukemic HL-60 cells were obtained from Y. Seino of the Okayama University School of Medicine. The cells were maintained in continuous culture in RPMI-1640 medium (Nissui Seiyaku Co., Ltd, Tokyo, Japan) supplemented with 10% dextran-coated charcoal-treated fetal calf serum (Gibco BRL, Grand Island, NY, USA), and kanamycin (0.06 mg/ml; Sigma) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells (1×10^7) were suspended in 800 µl of the serum free medium and electropolated with 10 µg luciferase reporter plasmid (pGVB2 vector, Toyo Ink Co., Ltd, Tokyo, Japan) inserted with a rat 25(OH)D$_3$-24-hydroxylase gene promoter (–291/+9) including the two VDREs and 5 µg of the pRL-CMV vector (Toyo Ink Co., Ltd) as an internal control using GENE PULSER II (Bio-Rad Laboratories, Hercules, CA). Cells were cultured for 24 h in the RPMI1640 medium containing of 10% FCS at 37°C in a humidified atmosphere of 5% carbon dioxide in air. After 24 h, cells were seeded at 210^5 cells/well in 24-well plates, and incubated for 2 days with between 10^{-8} M of 1α,25(OH)$_2$D$_3$ or an analog. The luciferase activities of the cell lysates were measured with a luciferase assay system (Toyo Ink Co., Ltd), according to the manufacturer's instructions. Transactivation measured by luciferase activity was standardized with the luciferase activity of the same cells determined by the Sea Pansy luciferase assay system as a control (Toyo Ink Co., Ltd). Each set of experiments was repeated at least 3×, and the results are presented in terms of fold-induction as means standard errors.

HL-60 cells and synchronization of cell cycle at S phase by excess amounts of thymidine

The doubling time of HL-60 cells was ~24 h. The cells were synchronized at S phase by the following procedure: cells (410^5 cells/ml) were cultured in 30 ml of RPMI-1640 medium for 24 h and subsequently cultured for 16 h in RPMI-1640 medium supplemented with 2.5 mM thymidine. After washing the cells with Ca,Mg-free phosphate buffered saline (PBS(–)) twice, the synchronization of cell cycle by the same manner was repeated, and the cells thus obtained were used in the following experiments.

^{3}H-thymidine incorporation assay

Cells (10^5 cells/well) were placed in 24-well tissue culture plates, and cultured for 3 days with or without different concentrations of 1α,25(OH)$_2$D$_3$ or analogs. After washing with RPMI-1640 medium, the cells were incubated with [methyl-^{3}H]-thymidine (1 mCi/ml) in RPMI-1640 for 1 h under the same conditions and subsequently incubated for 5 min at room temperature in PBS(–) containing 100 mM unlabeled thymidine. Cells were dissolved in 0.1 M NaOH, and aliquots were taken for measuring radioactivity in a liquid scintillation counter.

Flow cytometry

Cells (10^5 cells/well) were placed in 24-well tissue culture plates and cultured for 3 days in RPMI-1640 medium with 10^{-7} M 1α,25(OH)$_2$D$_3$ or analogs under the same conditions as above. To reduce the effects of contact inhibition, control cells were adjusted to 60–70% confluency at the time of FACS analysis. Each group of cells was collected and washed with PBS(–) once. Then, the cells were resuspended in PBS(–) containing 0.2% Triton-X and 100 µg RNase, and incubated at 37°C for 1 h. Cells were washed with PBS(–) and incubated with 0.5 ml of DNA-staining solution containing propidium iodide (50 µg/ml) at 4°C for 20 min. The cells were analysed with a flow cytometer euipped with an argon laser (488 nm, Becton Dickinson FACScan™) and cell cycle distribution was analyzed by ModiFiT LT(Verity). Experiments were repeated 3×.

Cell surface antigen expression analysis

Cells (10^5 cells/well) were placed in 24-well tissue culture plates, and cultured for 3 days in RPMI-1640 medium with 10^{-7} M 1α,25(OH)$_2$D$_3$ or analogs under the same conditions as above. Each group of cells was then collected and washed with PBS(–) once. Then, the cells (210^5 cells) were resuspended in 100 µl diluent solution containing 1% bovine serum albumin (BSA) and 1% sodium azide and incubated with 10 µl human monoclonal FITC conjugated CD11b antibody (Sigma, USA) and 10 µl human monoclonal PE conjugated CD14 antibody (Sigma, USA) for 30 min at room temperature without light. The cells were washed once with Diluent solution and then fixed in 300 µl of PBS(–) containing 2% paraformaldehyde. Fluorescence was detected on a Becton Dickinson FACScan™ at excitation wavelength of 490 nm and emission wavelength of 520 nm. Results were recorded as the mean fluorescence index, which is the product of the % fluorescence and the mean fluorescence intensity, with 10^4 cells being counted per treatment.

Nuclear staining assay

Cells (10^5 cells/well) were placed in 24-well tissue culture plates, and cultured for 120 h in RPMI-1640 medium with 10^{-7} M 1α,25(OH)$_2$D$_3$ or analogs under the same conditions as above. Each group of cells was then collected and washed with PBS(–) once. The cells were fixed with 1% glutaraldehyde in PBS(–) for 30 min, then washed with PBS(–). The preparations were suspended in PBS(–) and stained with 1 mM Hoechst33258 (Calbiochem) in PBS(–) for 10 min. The cells were viewed under an OLYMPUSIX70 microscope. Condensed and fragmented nuclei were counted, and percentages were calculated. Three random fields of view were observed with a minimum number of 200 cells scored per field.

Tdt-mediated dUTP nick end-labeling (TUNEL) assay

Cells (10^5 cells/well) were placed in 24-well tissue culture plates, and cultured for 120 h in RPMI-1640 medium with 10^{-7} M 1α,25(OH)$_2$D$_3$ or analogs under the same conditions as above. Each group of cells was then collected and washed with PBS(–) once. The cells were fixed with 4% paraformaldehyde in PBS(–) for 30 min and then washed with PBS(–). DNA fragmentation was detected by the *In Situ* Apoptosis Detection Kit (Takara Shuzo Co., Ltd, Japan) according to the manufacturer's protocol. This assay labels individual cells undergoing apoptosis by terminal transferase-mediated addition of fluorescein dUTP at DNA strand breaks. Following washing and mounting, cells were viewed under an OLYMPUS IX70 microscope and photographed.

DNA fragmentation assay

For assessment of quantitative DNA fragmentation (laddering), DNA was isolated from cells of each culture, and was examined for fragmentation. DNA was electrophoresed in a 2% agarose gel that was stained with ethidium bromide for observation under ultraviolet light [51,52].

Statistics

The results are expressed as means SEM. A paired or unpaired Student's t test was used to determine the significance of difference; a value of $P < 0.05$ was considered significant.

Supplementary material

Supplementary material including synthesis of analogs is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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