

# Calcitriol derivatives with two different side-chains at C-20. Part 4: Further chain modifications that alter VDR-dependent monocytic differentiation potency in human leukemia cells<sup>☆</sup>

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**Abstract**—Signaling of cell differentiation is one of the important physiological functions of the activated vitamin D receptor (VDR). Activation of the VDR can be achieved not only by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25D), the natural ligand, but also by a large number of its analogs. These include a category containing two side chains emanating at C-20, generally referred to as Gemini. The introduction of a cyclopropyl moiety as part of the pro-R side chain provides modified Gemini compounds with increased steric requirement and decreased chain flexibility; the biological consequences of this novel structural variant are subject of this investigation. In general, the resulting 1 $\alpha$ ,25-dihydroxy-(4-hydroxy-4-methyl-pentyl)-21,22-*cis*-cyclo-cholecalciferols reduced had differentiation and transcriptional potency and induced cell cycle arrest less efficiently, as shown by a decrease in G1/S ratio, when compared to 1,25D. Modifying their calcitriol side chain in the form of a 4-hydroxy-4-trifluoromethyl-5,5,5-trifluoropent-2-ynyl moiety, however, resulted in pronounced induction of differentiation in 1,25D-sensitive and moderate level of differentiation in 1,25D-resistant leukemia cells.

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## 1. Introduction

A number of modifications of the 1,25D molecule enhance its ability to activate VDR in various cell types.<sup>2</sup> These changes are designed to retard the degradation of the ligand within the target cells, and to induce an active conformational change of the VDR. Previous studies of calcitriol derivatives demonstrated that the introduction of the C-16 double-bond in the D-ring affects the conformation of the side-chain, improves the biological stability by arresting the metabolic degradation at the 24-keto stage,<sup>3</sup> generally enhances differentiation and anti-proliferative activity,<sup>4</sup> and reduces the hypercalcemic potential.<sup>5</sup> Structural modifications of the side-chain itself also interfere with the C-24 hydroxylation-initiated cascade of degradation and modify biological activities.<sup>6–8</sup> These include C-23-yne or

C-23(*E,Z*)-ene modifications and 26,27-hexafluorination. To reduce the inactivating C-3 epimerization, the A-ring can be modified as the 19-nor variant,<sup>9</sup> or by changing the geometry of the 5E double bond;<sup>10</sup> as a result, biological activities are enhanced.<sup>11,12</sup>

The discovery that 20-epi vitamin D analogs can exhibit higher potencies<sup>13,14</sup> engendered the synthesis of Gemini, distinguished by two identical side chains emanating at C-20.<sup>15,16</sup> In a second generation of Gemini compounds, chain-modifications aimed at metabolic retardation and enhanced differentiation were introduced stereoselectively<sup>17,18</sup> and their effects evaluated.<sup>19,20</sup>

Increased anti-proliferative activities of 20-desmethyl-20-cyclopropyl-cholecalciferol analogs have also been reported,<sup>21</sup> and an additional modification in the form of an unsaturated chain containing terminal trifluoromethyl groups and a 19-nor A-ring<sup>22</sup> has demonstrated enhanced transcriptional activity while maintaining low hypercalcemia-induction potential when compared to 1,25D.<sup>23</sup>

It stands to reason, therefore, that a combination of the 20-desmethyl-20-cyclopropyl cholecalciferols with the

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<sup>☆</sup> Ref. 1.

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Gemini counterparts would be of interest. Thus, the 20-desmethyl-20 cyclopropyl compounds were transmuted by the addition of a 2-hydroxy-2-methyl-butyl group to the cyclopropyl moiety so that this new side chain would be of the same length as that of calcitriol. This report presents the synthesis and biological evaluation of this new class of Gemini by studying their effect on HL60 cells,<sup>24</sup> a laboratory model of acute myeloid leukemia (AML).

## 2. Results

### 2.1. Synthesis

The key intermediate **2** prepared from alkene **1** has been described,<sup>17</sup> A Simmons–Smith reaction of **2** (Scheme 1) produced **3** stereospecifically in excellent yield.

Oxidation of the primary hydroxyl group with pyridinium chlorochromate (PCC) followed by a Wittig–Horner reaction gave the unsaturated ester **4**. The double bond in **4** was then hydrogenated without perturbing the cyclopropyl ring. The resulting intermediate was treated with methylmagnesium bromide to afford the crystalline diol **5**. An ORTEP drawing of **5**, revealing the absolute configuration, is shown in Figure 1.

The silyl ether protective group in **5** was cleaved with aqueous fluorosilicic acid<sup>25</sup> and the resulting triol **6** was oxidized with pyridinium dichromate (PDC) and the tertiary hydroxyl groups protected as trimethylsilyl ethers. Wittig–Horner reactions of the resulting ketone **7** with **8**, **9**, and **10**, followed by treatments with tetrabutylammonium fluoride, gave **11**, **12**, and **13**, respectively, as illustrated in Scheme 2.

The aldehyde obtained from diol **3** was homologated with (1-diazo-2-oxopropyl) phosphonic acid dimethyl

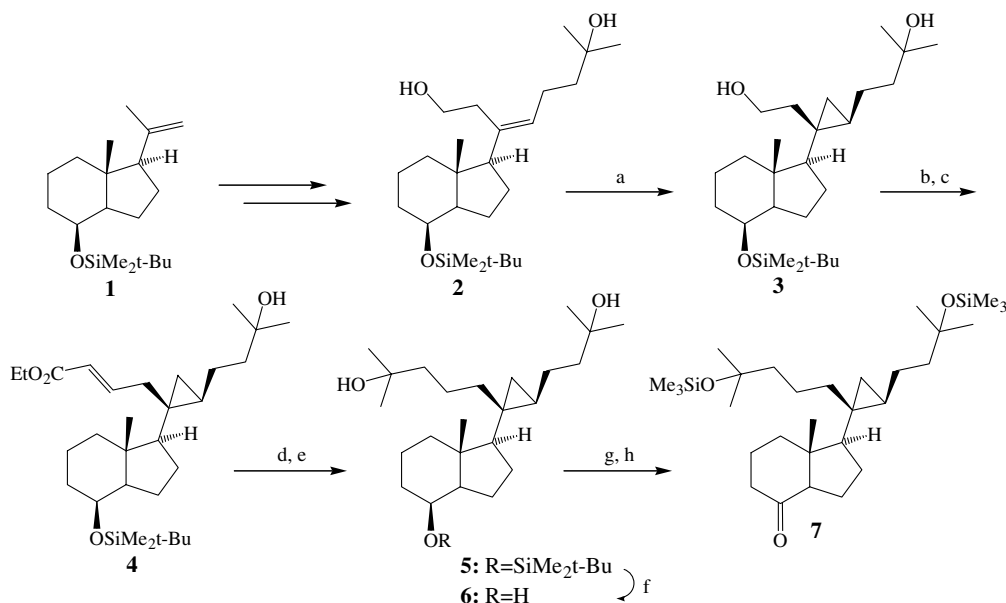
ester to the acetylene **14** then protected with a trimethylsilyl group to generate **15** as illustrated in Scheme 3. A subsequent condensation with hexafluoroacetone gave **16**. Removal of the silyl groups, followed by oxidation of the resulting alcohol with PDC, gave ketone **18** and Wittig–Horner reactions of **18** with **8** and **9** led to **19** and **20** as shown in Scheme 4.

### 2.2. Biological evaluations

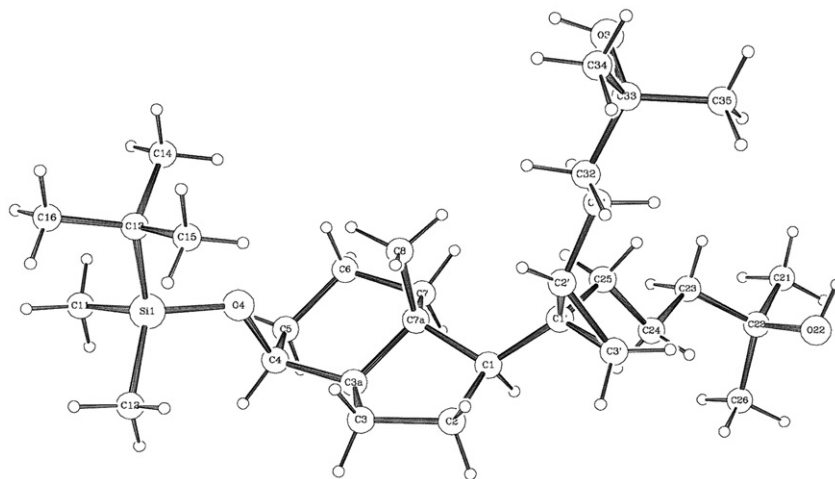
The effect of these chemical modifications on monocytic differentiation-inducing potency was studied and revealed that the 21,22-cyclo modification of Gemini as in **11** had little effect on the induction of markers of differentiation. Indeed, **11** was ca. 16-fold less potent at inducing CD14 and CD11b differentiation markers than 1,25D as shown in Table 1 and Figure 2.

In agreement with previous reports on A-ring-modified Gemini,<sup>26</sup> additional A-ring modifications of **11** reduced the potency even more; the 19-nor derivative **12** had less than half the activity, while the substitution of the 1 $\alpha$ -hydroxyl group with a fluorine atom as in **13** abolished the differentiation-inducing activity. Hexafluorination at C-26 and C-27, together with the insertion of a triple bond as in **19**, however, increased the differentiation-inducing activity when compared with **11** but the corresponding 19-nor analog **20** exhibited slightly reduced potency.

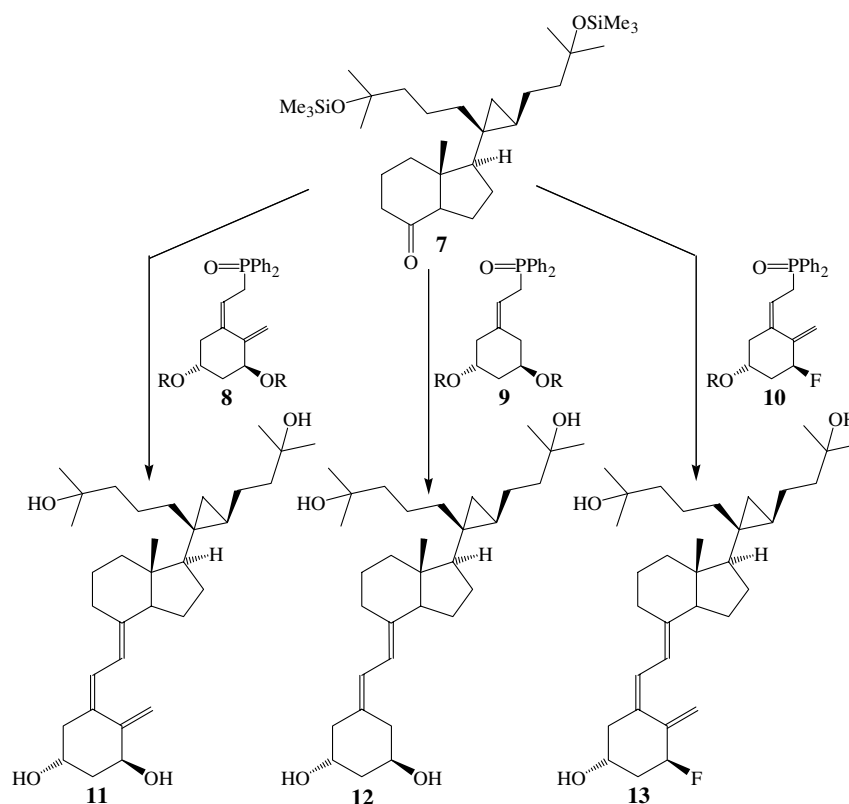
Cell cycle analyses were performed to investigate the ability of these compounds to inhibit the cell cycle progression, which is determined by an increase in the number of cells in G1 phase (G1 arrest). Figure 3 illustrates the ratio of cells in G1 phase to S phase (G1/S). Compound **11** inhibited cell cycle progression only minimally and changes in the A-ring as in **12** and **13** did not enhance this activity. Only modest cell cycle inhibition activities were observed when a side chain was equipped



**Scheme 1.** Reagents: (a) Et<sub>2</sub>Zn, CH<sub>2</sub>I<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>; (b) PCC, CH<sub>2</sub>Cl<sub>2</sub>; (c) EtO<sub>2</sub>P(O)CH<sub>2</sub>COOEt, *t*-BuOK, toluene; (d) H<sub>2</sub>, Pd/C, AcOEt; (e) MeMgBr, Et<sub>2</sub>O; (f) H<sub>2</sub>SiF<sub>6</sub>, THF–CH<sub>3</sub>CN; (g) PDC, CH<sub>2</sub>Cl<sub>2</sub>; (h) TMS-imidazole, CH<sub>2</sub>Cl<sub>2</sub>.



**Figure 1.** X-ray structure of molecule **5** at 100 °C.



**Scheme 2.**

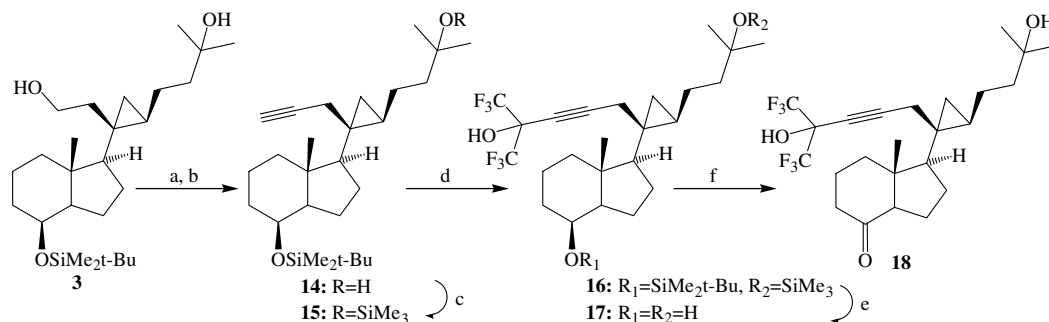
with molecular features generally regarded as metabolic stabilizers, as in **19** and **20**.

A concern regarding the potential clinical use of vitamin D analogs for the treatment of cancer is the development of resistance to their differentiation-inducing effects. Thus, we studied the ability to overcome the resistance to differentiation in HL60-40AF (40AF) cells adapted to grow in the presence of 1,25D.<sup>27</sup> The Gemini compounds **19** and **20** with side-chain fluorination and unsaturation were particularly effective in inducing differentiation in these 1,25D-resistant cells (Fig. 4). Future

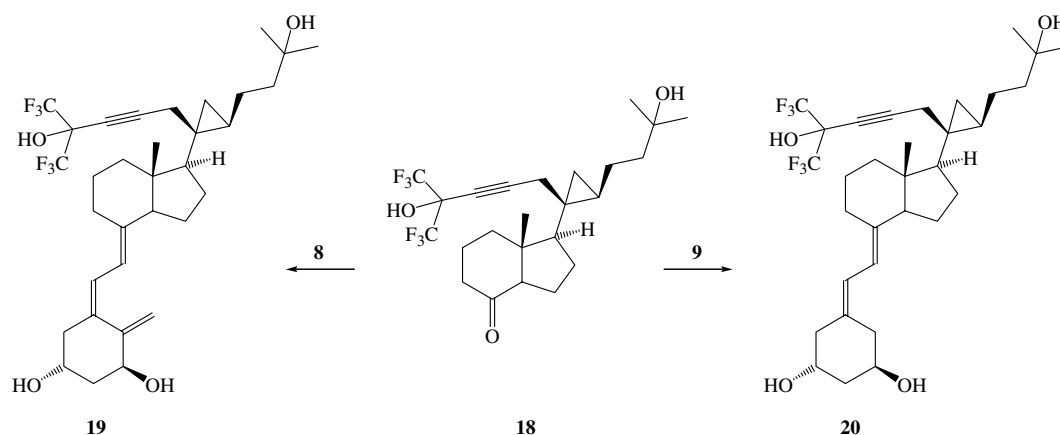
studies should determine if these compounds have sufficiently low toxicity to warrant further development as antineoplastic agents.

### 2.3. VDR transcriptional activities

To determine the correlation between the effects of the new Gemini on differentiation and on the induction of VDR-transcription activity, we utilized quantitative PCR (qPCR) to measure the mRNA levels of CYP24 which is a major VDR target gene. Figures 5 and 6 illustrate the relative quantitation of CYP24 mRNA, nor-



**Scheme 3.** Reagents: (a) PCC, DCM; (b) MeCON<sub>2</sub>P(O)(OMe)<sub>2</sub>, Na<sub>2</sub>CO<sub>3</sub>, MeOH; (c) TMS-imidazole, DCM; (d) (CF<sub>3</sub>)<sub>2</sub>CO, BuLi, THF; (e) Bu<sub>4</sub>NF, THF; (f) PDC, DCM.



**Scheme 4.**

**Table 1.** Effect on the expression of differentiation markers in HL60-G cells following 48-h treatment, denoted as ED<sub>20</sub> values

| Compound  | CD 11b<br>(nM)    | CD 14<br>(nM)     | CD 11b <sup>a</sup> | CD 14 <sup>a</sup> |
|-----------|-------------------|-------------------|---------------------|--------------------|
| 1,25D     | 1.20              | 0.57              | 1.00                | 1.00               |
| <b>11</b> | 19.75             | 9.97              | 0.06                | 0.06               |
| <b>12</b> | 98.75             | 66.03             | 0.01                | 0.009              |
| <b>13</b> | >500 <sup>b</sup> | >500 <sup>a</sup> | <0.002              | <0.001             |
| <b>19</b> | 0.52              | 0.24              | 2.31                | 2.38               |
| <b>20</b> | 1.17              | 0.46              | 1.03                | 1.24               |

<sup>a</sup> Relative potency (shaded) with respect to 1,25D.

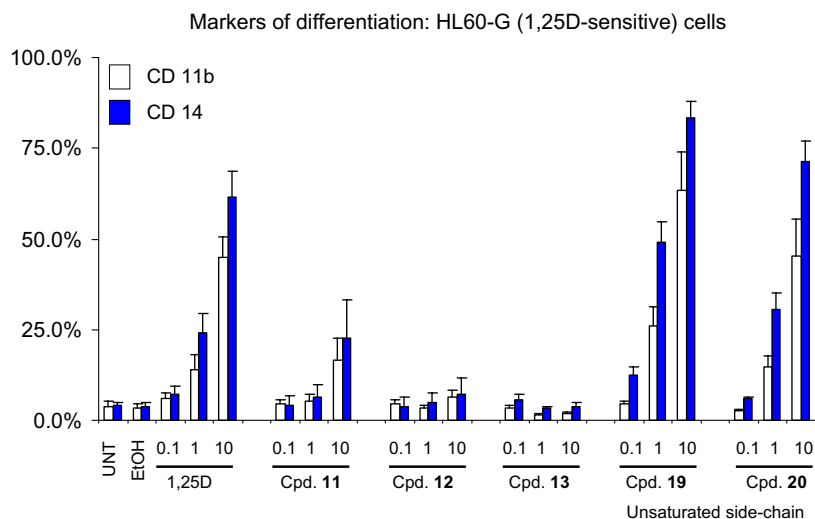
<sup>b</sup> Indicates projected ED<sub>20</sub> values derived from data.

malized to the levels induced by 1,25D. In HL60-G cells, compound **11** did not induce CYP24 transcription after a 12-h treatment at 1 nM concentration, a result that is in agreement with its poor differentiating potency. Similarly, **12** and **13** modifications did not improve transcriptional effectiveness, again in agreement with their differentiation-inducing capacity and prior studies with A-ring-modified Gemini.<sup>26</sup> A 4-hydroxy-4-trifluoromethyl-5-trifluoro-pent-2-ynyl side chain, as in **19**, propelled the levels of CYP24 mRNA above the levels induced by the normal ligand. While **19** exhibited a ca. 30-fold greater transcriptional potency than 1,25D, the 19-nor A-ring analog **20** was only ca. 2.5-fold as active as 1,25D.

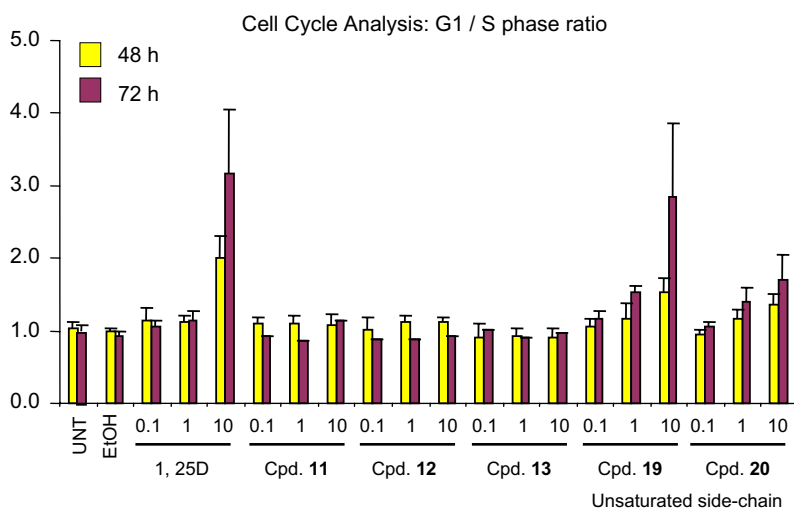
Studies in 1,25D-resistant 40AF cells indicated that hexafluorinated and unsaturated analogs were superior to 1,25D in the induction of CYP24 mRNA (Fig. 6), with a similar relative potency as in HL60-G cells. These data demonstrate that increased transcriptional activity of VDR is associated with the increased potency to induce differentiation in human myeloid leukemia cells.

### 3. Discussion

These studies are a part of our attempts at side-chain optimization of Gemini and the resulting ligand-induced conformational changes in the VDR.<sup>28,30,31</sup> Previous studies have shown that Vitamin D analogs of the Gemini type can enhance the biological activity of the VDR.<sup>14,15,28</sup> However, the introduction of a cyclopropyl arrangement to the pro-R calcitriol side chains of Gemini did not enhance their biological activity in HL60 cells relative to 1,25D, neither did additional A-ring modifications, and, in some cases, abolished their biological activities. A possible explanation may be found in the difficulty to accommodate the steric restrictions imposed by the VDR ligand binding domain (LBD).<sup>29</sup> It is of interest to note that the marked enhancement of differentiation potencies by hexafluorination at C-26 and C-27 (Fig. 2) was not fully paralleled by the increased G1 arrest (Fig. 3). This is in keeping with a previous observation that differentiation of human leukemian



**Figure 2.** Analysis of the potency of novel cyclopropyl compounds to induce markers of monocytic differentiation. HL60-G cells expressed minimal amounts of CD11b and CD14 when left untreated or treated with EtOH. When treated with 1,25D a dose-dependent increase in the expression of CD11b and CD14 was observed with increasing concentrations (0.1, 1, and 10 nM). When the cells were treated with Gemini modified with a combination of C-20-cyclopropyl, as well as structures that protected the molecule from side-chain and A-ring metabolism, differentiation potency greater than 1,25D was observed.



**Figure 3.** Analysis of cell cycle regulation. HL60-G cells were treated with increasing drug concentrations for 48 and 72 h. Marked induction of G1 arrest, demonstrated by an increase in the fraction of cells in the G1-phase over cells in S-phase (G1/S ratio), was observed in cells treated with **19**. A modest increase in G1/S was observed in the less potent **20**.

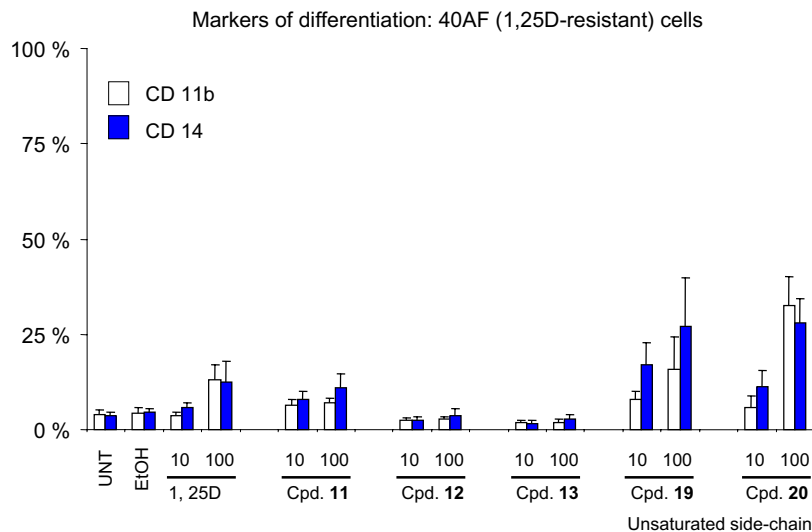
cells can be partially uncoupled from cell cycle arrest,<sup>32</sup> and raises the possibility that fluorination of Gemini has greater pro-differentiation than anti-proliferation activity.

Compounds **19** and **20** were particularly effective in overcoming resistance of differentiation of 40AF cells (Fig. 4), but curiously this did not strictly correlate with transcriptional activation of the VDR target gene, CYP24 (Fig. 6). For instance, compound **12** had no ability to induce differentiation, but it did induce CYP24 mRNA expression to the same extent as compound **20**, which has the most effect in inducing 40AF cell differentiation among the analogs tested

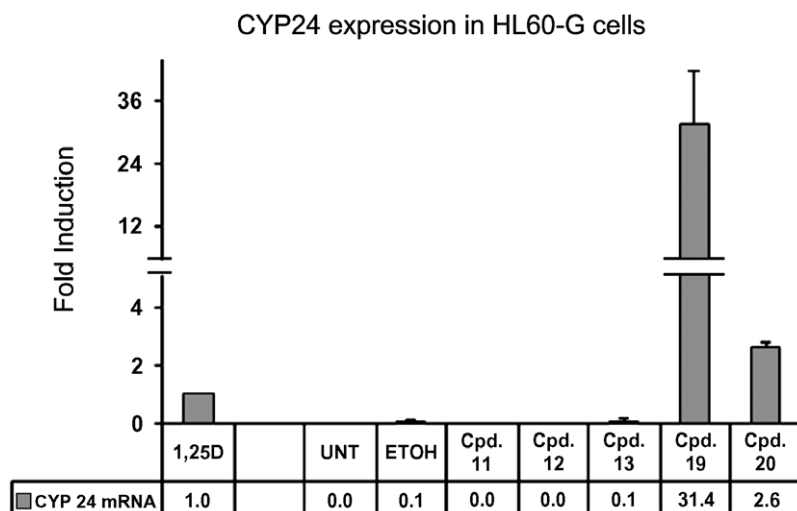
here. This suggests that the lack of transcriptional activation of VDR in 40AF cells is only one of the factors responsible for resistance to differentiation of these cells. Thus, judicious use of vitamin D analogs may help to unravel differentiation-related pathways in malignant human cells.

#### 4. Conclusions

The studies presented here focus on structural modifications of Gemini that influence the differentiation-inducing, anti-proliferative, and transcriptional activity of the compounds in human leukemia cells.



**Figure 4.** Analysis of the potency of novel cyclopropyl deltanoids to reverse resistance to 1,25D in a HL60-G cell variant. 40AF cells expressed minimal amounts of CD11b and CD14 when left untreated or treated with EtOH, the vehicle for calcitriol, and derivatives. When treated with high concentration of 1,25D, a minimal increase in the expression of CD11b and CD14 differentiation markers was observed with increasing concentrations (10 and 100 nM). A modest reversal of resistance was observed when these cells were treated with **19** and **20**.



**Figure 5.** Induction of CYP24 mRNA by novel 1,25D analogs. HL60-G cells were treated for 12 h with 1 nM drug concentration. CYP24 transcript was quantitated by a Lightcycler® based, Syber green realtime PCR assay. Acidic Riboprotein Subunit 0 (ARP0) was utilized as an internal control and all samples were normalized to 1,25D treatment. Compound **19** showed an exceptionally high induction of CYP24.

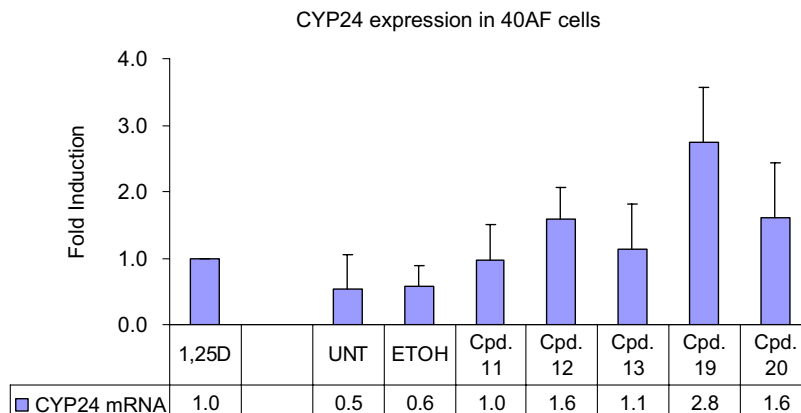
The cyclopropyl modification at the pro-R side-chain decreased the activity of the compound compared to 1,25D, and further A-ring modifications did not restore this activity. When the cyclopropyl modification was combined, at the pro-S side chain of Gemini, with established changes aimed at protection against metabolic degradation, the biological activity of the resulting compound exceeded the activity of 1,25D and was characterized, in part, by an increased level of VDR-induced transcriptional activity. These in vitro studies further suggest the directions for additional structural modifications of calcitriol derivatives aimed at improving the potencies for differentiation and proliferation arrest of malignant cells.

## 5. Experimental

### 5.1. 4-[(1*R*,2*S*)-2-[(1*S*,3*aR*,4*S*,7*aR*)-4-(*tert*-Butyl-dimethyl-silanyloxy)-7*a*-methyl-octahydro-inden-1-yl]-2-(2-hydroxy-ethyl)-cyclopropyl]-2-methyl-butan-2-ol (**3**)

A 1 M solution of diethylzinc in hexane (4.30 mL) was added slowly at  $-22^{\circ}\text{C}$  to dichloromethane (8 mL) and then diiodomethane (1.21 g, 4.50 mmol) in dichloromethane (0.5 mL) was added dropwise. The mixture was allowed to stir for 15 min before a solution of **2** (376 mg, 0.89 mmol) in dichloromethane (2 mL) was added. The mixture was stirred for 3 h at  $-20^{\circ}\text{C}$  and for 3.5 h at room temperature. The mixture was quenched by addition of a saturated soln of ammonium chloride (20 mL)





**Figure 6.** Induction of CYP24 mRNA in cells resistant to 1,25D-induced differentiation. 40AF cells were treated for 12 h with 1 nM drug concentration. Relative quantitation of CYP24 transcript was performed using a Lightcycler® based, Syber green PCR assay. Acidic Riboprotein Subunit 0 (ARP0) was utilized as the internal control and all samples were normalized to 1,25D treatment. In this model of acute myeloid leukemia (AML), which is highly resistant to the chemotherapeutic properties of 1,25D, **19** and **20** induced levels of CYP24 that exceeded those induced by 1,25D.

at 0 °C then 1 M H<sub>2</sub>SO<sub>4</sub> (10 mL) was added. The mixture was extracted three times with dichloromethane (30 mL each). The combined organic layers were washed with brine (30 mL) then dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated. The oily residue was flash-chromatographed using 3:2 → 1:1 hexane/ethyl acetate as mobile phase to give 341 mg (88%) of **3** as a colorless oil; H NMR (CDCl<sub>3</sub>) δ 3.97 (1H, br s), 3.94–3.86 (1H, m), 3.81–3.72 (1H, m), 2.19–2.11 (1H, m), 2.01 (1H, d, *J* = 12.1 Hz), 1.90–1.81 (2H, m), 1.68–1.15 (15H, m), 1.22 (6H, s), 1.02–0.85 (2H, m), 0.94 (3H, s), 0.89 (9H, s), 0.40 (1H, dd, *J* = 9.2, 4.0 Hz), 0.00 (3H, s), −0.01 (3H, s), −0.24 to −0.27 (1H, m).

**5.2. 4-[(1*S*,2*R*)-1-[(1*S*,3*aR*,4*S*,7*aR*)-4-(*tert*-Butyl-dimethyl-silanyloxy)-7*a*-methyl-octahydro-inden-1-yl]-2-(3-hydroxy-3-methyl-butyl)-cyclopropyl]-but-2-enoic acid ethyl ester (**4**)**

To the suspension of pyridinium chlorochromate (1.18 g, 5.47 mmol) and Celite (1.19 g) in dichloromethane (15 mL) was added dropwise **3** (1.20 g, 2.74 mmol) in dichloromethane (6 mL) and the mixture was stirred at room temperature for 1 h. Reaction mixture was filtered through a column with silica gel (50 mL) and a layer of Celite (2 cm) and the column was washed with dichloromethane and 10:1 dichloromethane/ethyl acetate. The filtrate was evaporated to furnish the crude aldehyde. A 1 M soln of potassium *tert*-butoxide in tetrahydrofuran (5.60 mL) was diluted with toluene (15 mL), cooled to 5 °C, and a soln of triethyl phosphonoacetate (1.26 mg, 5.62 mmol) in toluene (1.5 mL) was added dropwise. The mixture was stirred at room temperature for 1 h, then cooled to −15 °C and a soln of the aldehyde in toluene (4 mL) was added. Stirring was continued at −10 °C for 2.5 h. The reaction mixture was quenched by the addition of saturated solution of ammonium chloride (50 mL). The mixture was diluted with toluene (100 mL) and the organic layer was washed twice with water (25 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated. The residue was purified by flash chromatography using 6:1 hexane/ethyl acetate as mobile phase to

give 996 mg (72%) of **4** as a colorless oil; H NMR (CDCl<sub>3</sub>) δ 7.09–6.97 (1H, m), 5.86 (1H, br d, *J* = 16.1 Hz), 4.20 (2H, q, *J* = 7.1 Hz), 3.99–3.94 (1H, m), 2.81–2.71 (1H, m), 2.00–1.92 (1H, m), 1.88–1.74 (2H, m), 1.68–1.10 (14H, m), 1.28 (3H, t, *J* = 7.2 Hz), 1.23 (6H, s), 1.02–0.86 (2H, m), 0.95 (3H, s), 0.89 (9H, s), 0.50–0.44 (1H, m), 0.00 (3H, s), −0.02 (3H, s), −0.21 to −0.26 (1H, m).

**5.3. 5-[(1*R*,2*R*)-1-[(1*S*,3*aR*,4*S*,7*aR*)-4-(*tert*-Butyl-dimethyl-silanyloxy)-7*a*-methyl-octahydro-inden-1-yl]-2-(3-hydroxy-3-methyl-butyl)-cyclopropyl]-2-methyl-pentan-2-ol (**5**)**

A solution of **4** (996 mg, 1.97 mmol) in ethyl acetate (20 mL) was hydrogenated over 10% Pd/C (185 mg) at ambient temperature and atmospheric hydrogen pressure. The reaction was monitored by TLC (6:1 hexane/ethyl acetate). After 2 h the catalyst was filtered off and the filtrate evaporated to give a colorless oil (980 mg). The residue was dissolved in diethyl ether (15 mL) and cooled in an ice bath. A 3 M methylmagnesium bromide in diethyl ether (3.85 mL, 11.556 mmol) was added dropwise. After completion of the addition the mixture was stirred at room temperature for 3.5 h then cooled in an ice bath and a saturated solution of ammonium chloride (10 mL) was added dropwise. The resulting precipitate was dissolved by the addition of water (50 mL). The aqueous layer was re-extracted three times with diethyl ether (30 mL) and the combined ether layers were dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated. The oil residue was flash-chromatographed using 2:1 hexane/ethyl acetate as mobile phase. The fractions containing mixtures (ca. 400 mg) were re-chromatographed. Fractions containing pure product were pooled and evaporated to a crystalline residue that was re-crystallized from hexane to give **5** (815 mg, 84%); mp 123–124 °C; H NMR (CDCl<sub>3</sub>) δ 4.00–3.95 (1H, m), 2.20–1.96 (2H, m), 1.87–1.75 (2H, m), 1.70–1.18 (19H, m), 1.23 (6H, s), 1.22 (6H, s), 1.02–0.77 (2H, m), 0.92 (3H, s), 0.89 (9H, s), 0.35–0.31 (1H, m), 0.00 (3H, s), −0.01 (3H, s), −0.39 to −0.42 (1H, m).

#### 5.4. (1*S*,3*aR*,4*S*,7*aR*)-1-[(1*R*,2*R*)-2-(3-Hydroxy-3-methyl-butyl)-1-(4-hydroxy-4-methyl-pentyl)-cyclopropyl]-7*a*-methyl-octahydro-inden-4-ol (**6**)

A solution of aqueous fluorosilicic acid (3 mL) was added dropwise to a solution of **5** (767 mg, 1.55 mmol) in acetonitrile (20 mL) and tetrahydrofuran (5 mL). The mixture was stirred for 1.5 h, then two portions of  $\text{H}_2\text{SiF}_6$  (1 mL) were added, the second (0.5 mL) 75 min later. The mixture was stirred for 1 h, the resulting mixture was diluted with water (15 mL) then poured into a mixture of ethyl acetate (125 mL) and water (60 mL). The aqueous phase was re-extracted three times with ethyl acetate (75 mL). The combined organic layers were dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated. The oil residue was first filtered through silica gel (25 mL), filtrate and ethyl acetate washings were evaporated and flash-chromatographed using 1:1 hexane/ethyl acetate as mobile phase to give **6** (430 mg, 73%) as a colorless oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.07–4.04 (1H, m), 2.07–1.98 (2H, m), 1.87–1.75 (3H, m), 1.70–1.13 (19H, m), 1.23 (6H, s), 1.22 (6H, s), 1.02–0.78 (2H, m), 0.96 (3H, s), 0.34 (1H, dd,  $J = 8.8, 4.4$  Hz),  $-0.36$  to  $-0.39$  (1H, m).

#### 5.5. (1*S*,3*aR*,7*aR*)-7*a*-Methyl-1-[(1*R*,2*R*)-2-(3-methyl-3-trimethylsilanyloxy-butyl)-1-(4-methyl-4-trimethylsilanyloxy-pentyl)-cyclopropyl]-octahydro-inden-4-one (**7**)

A solution of **6** (430 mg, 1.13 mmol) in dichloromethane (5 mL) was added dropwise to the suspension of pyridinium dichromate (1.40 g, 3.61 mmol) and Celite (1.5 g) in dichloromethane (15 mL). The mixture was stirred at room temperature for 4 h and additional pyridinium dichromate (400 mg, 1.06 mmol) was added. After 1 h the reaction mixture was filtered through a column with silica gel, the filter was washed with dichloromethane and ethyl acetate then evaporated to give the crude ketone as an oil (405 mg). This material was dissolved in dichloromethane (12 mL) and 1-(trimethylsilyl)imidazole (1.26 mL) was added. The mixture was stirred at room temperature for 2 h, diluted with water (10 mL), and stirred for 10 min and then water (75 mL) was added. The aqueous layer was extracted four times with ethyl acetate (40 mL each). The combined organic layers were washed with brine (30 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The resulting oily residue was flash-chromatographed using 20:1 hexane/ethyl acetate as mobile phase to give **7** as colorless oil (472 mg, 84%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 2.50 (1H, dd,  $J = 11.2, 7.1$  Hz), 2.36–2.12 (4H, m), 2.07–1.86 (2H, m), 1.83–1.61 (4H, m), 1.56–0.98 (9H, m), 1.24 (6H, s), 1.20 (6H, s), 0.91–0.78 (3H, m), 0.67 (3H, s), 0.36 (1H, dd,  $J = 9.2, 5.1$  Hz), 0.12 (9H, s), 0.10 (9H, s),  $-0.33$  (1H, t,  $J = 5.1$  Hz).

#### 5.6. 1*α*,25-Dihydroxy-20*R*-(4-hydroxy-4-methyl-pentyl)-21,22*R*-cyclo-cholecalciferol (**11**)

A 1.6 M butyllithium in hexane (0.420 mL, 0.673 mmol) was added dropwise to a solution of **8** (392 mg, 0.67 mmol) in tetrahydrofuran (6 mL) at  $-78^\circ\text{C}$ . The flask was protected from light and the resulting deep red solution was stirred at  $-78^\circ\text{C}$  for 10 min then **7**

(150 mg, 0.287 mmol) in tetrahydrofuran (1.5 mL) was added dropwise. The reaction mixture was stirred for 2 h, removed from the bath, and then poured into hexane (50 mL) and brine (50 mL). The water fraction was extracted three times with hexane (40 mL), dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The oil residue was chromatographed on column (25 mL, protected from light) using 20:1 hexane/ethyl acetate as mobile phase. Fractions containing the product were pooled and evaporated to give colorless oil, which was treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (4 mL). The reaction mixture was stirred at room temperature for 8.5 h. The mixture was dissolved by the addition of ethyl acetate (100 mL) and washed six times with water (40 mL) and brine (20 mL), dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The oil residue was flash-chromatographed using ethyl acetate as mobile phase. Fractions containing product were pooled and evaporated to give a colorless oil product, which was dissolved in methyl acetate (20 mL) and evaporated (4 times) to give 135 mg (91%) of **11** as white foam,  $[\alpha]_{\text{D}}^{30} -41.0$ , ( $c$  0.41,  $\text{CHCl}_3$ ); UV  $\lambda_{\text{max}}$  (EtOH) 213 nm ( $\epsilon$  14481), 265 nm ( $\epsilon$  16200);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.36 (1H, d,  $J = 11.3$  Hz), 5.97 (1H, d,  $J = 11.3$  Hz), 5.32 (1H, s), 4.98 (1H, s), 4.42 (1H, br s), 4.22 (1H, br s), 2.83–2.79 (1H, m), 2.59 (1H, br d,  $J = 13.3$  Hz), 2.31 (1H, dd,  $J = 13.3, 6.2$  Hz), 2.20–2.16 (1H, m), 2.05–1.99 (3H, m), 1.93–1.87 (1H, m), 1.83–1.19 (18H, m), 1.23 (6H, s), 1.20 (6H, s), 1.07–0.77 (5H, m), 0.56 (3H, s), 0.37–0.34 (1H, m),  $-0.34$  to  $-0.37$  (1H, m).

#### 5.7. 1*α*,25-Dihydroxy-20*R*-(4-hydroxy-4-methyl-pentyl)-21,22*R*-cyclo-19-nor-cholecalciferol (**12**)

Following the procedure for synthesis of **11**, 1.6 M butyllithium in hexane (0.51 mL, 0.82 mmol) was added to a solution of **9** (468 mg, 0.82 mmol) in anhydrous tetrahydrofuran (6 mL) and then **7** (140 mg, 0.27 mmol) in anhydrous tetrahydrofuran (1.6 mL) was added dropwise. After chromatography the oil residue was treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (6 mL). The reaction mixture was stirred at room temperature for 15 h. A new portion of 1 M tetrabutylammonium fluoride in tetrahydrofuran (4 mL) was added, and the solution was stirred for next 7 h. Workup and chromatography gave 105 mg (77%) of **12** as white foam.  $[\alpha]_{\text{D}}^{25} +3.60$  ( $c$  0.44,  $\text{CHCl}_3$ ), UV  $\lambda_{\text{max}}$  (EtOH): 243 nm ( $\epsilon$  32493), 252 nm ( $\epsilon$  38183), 261 nm ( $\epsilon$  26327).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 6.30 (1H, d,  $J = 11.3$  Hz), 5.81 (1H, dd,  $J = 11.3$  Hz), 4.11 (1H, br s), 4.05 (1H, br s), 2.81–2.70 (2H, m), 2.50–2.48 (1H, m), 2.24–2.17 (2H, m), 2.08–1.92 (2H, m), 1.83–1.77 (2H, m), 1.73–0.99 (20H, m), 1.23 (6H, s), 1.21 (6H, s), 0.93–0.76 (4H, m), 0.57 (3H, s), 0.39–0.36 (1H, m),  $-0.34$  (1H, dd,  $J = 5.1, 5.1$  Hz), HR-MS calculated for  $\text{C}_{32}\text{H}_{54}\text{O}_4 + \text{Na}$ : 525.3914; found: 525.3914.

#### 5.8. 1*α*-Fluoro-25-hydroxy-20*R*-(4-hydroxy-4-methyl-pentyl)-21,22*R*-cyclo-cholecalciferol (**13**)

Following the procedure for the synthesis of **11**, 1.6 M butyllithium in hexane (0.15 mL, 0.24 mmol) was added to a solution of **10** (113 mg, 0.24 mmol) in anhydrous



tetrahydrofuran (4 mL) and then **7** (55 mg, 0.11 mmol) in anhydrous tetrahydrofuran (1.3 mL) was added dropwise. After chromatography the oil residue was treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (3 mL). The reaction mixture was stirred at room temperature for 20 h. Workup and chromatography on column (25 mL, protected from light) using 1:1 hexane/ethyl acetate as mobile phase gave 24 mg (44%) of **13** as colorless oil; H NMR (CDCl<sub>3</sub>)  $\delta$  6.36 (1H, d,  $J$  = 11.3 Hz), 5.97 (1H, d,  $J$  = 11.5 Hz), 5.34 (1H, s), 5.12 (1H, br d,  $J$  = 42.5 Hz), 5.06 (1H, s), 4.20 (1H, br s), 2.82–2.76 (1H, m), 2.61–2.57 (1H, m), 2.30–2.85 (1H, m), 2.18–2.11 (2H, m), 2.05–1.92 (4H, m), 1.80–1.12 (15H, m), 1.20 (6H, s), 1.18 (6H, s), 1.99–0.79 (6H, m), 0.54 (3H, s), 0.35–0.32 (1H, m), –0.37 to –0.39 (1H, m).

**5.9. 4-[(1R,2S)-2-[(1S,3aR,4S,7aR)-4-(tert-Butyl-dimethyl-silanyloxy)-7a-methyl-octahydro-inden-1-yl]-2-prop-2-ynyl-cyclopropyl]-2-methyl-butan-2-ol (14)**

To the suspension of pyridinium chlorochromate (422 mg, 1.958 mmol) and Celite (430 mg) in dichloromethane (8 mL) **3** (397 mg, 0.91 mmol) in dichloromethane (5 mL) was added dropwise and the mixture was stirred at room temperature for 1 h. The reaction mixture was filtered through a column with silica gel (50 mL) and Celite (3 cm) using dichloromethane and 10:1 dichloromethane/ethyl acetate as mobile phases. The fractions containing product were pooled and evaporated to give oil. Oil was dissolved in methanol (4 mL) and 1-(diaz-2-oxo-propyl)-phosphonic acid dimethyl ester (261 mg, 1.36 mmol) in methanol (1 mL) was added. The resulting mixture was cooled in an ice bath and potassium carbonate (257 mg, 1.86 mmol) was added. The reaction mixture was stirred in an ice bath for 35 min and then at room temperature for 1.5 h. Water (50 mL) was added and the mixture was extracted three times with hexane (30 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated. The oil residue was chromatographed on column (100 mL) using 8:1 hexane/ethyl acetate as mobile phase. Fractions containing product were pooled and evaporated to give 245 mg (63%) of **14** as colorless oil; H NMR (CDCl<sub>3</sub>): 3.98 (1H, br s), 2.64 (1H, d,  $J$  = 18.3 Hz), 2.12 (1H, br t,  $J$  = 9.9 Hz), 2.05–1.80 (3H, m), 1.69–1.22 (14H, m), 1.22 (6H, s), 1.12–0.88 (2H, m), 0.93 (3H, s), 0.89 (0H, s), 0.47 (1H, dd,  $J$  = 9.0, 4.6 Hz), 0.00 (3H, s), –0.01 (3H, s), –0.09 (1H, br t,  $J$  = 4.9 Hz).

**5.10. (1S,3aR,4S,7aR)-4-(tert-Butyl-dimethyl-silanyloxy)-7a-methyl-1-[(1S,2R)-2-(3-methyl-3-trimethylsilanyloxy-butyl)-1-prop-2-ynyl-cyclopropyl]-octahydro-indene (15)**

A 1-(trimethylsilyl)imidazole (0.65 mL, 4.43 mmol) was added dropwise to the solution of **14** (460 mg, 1.06 mmol) in dichloromethane (12 mL). The mixture was stirred at room temperature for 50 min. Water (50 mL) was added and the mixture was extracted three times with hexane (50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated. The oil residue was chromatographed on column (120 mL) using 100:1 → 50:1 hexane/ethyl acetate as mobile phases. Fractions containing product were pooled and evaporated to give 484 mg (90%) of product **15** as colorless oil; H NMR (CDCl<sub>3</sub>): 3.98

(1H, br s), 2.65 (1H, br d,  $J$  = 18.7 Hz), 2.12 (1H, t,  $J$  = 9.2 Hz), 2.02–1.90 (3H, m), 1.86–1.62 (2H, m), 1.59–1.11 (9H, m), 1.20 (6H, s), 1.03–0.86 (4H, m), 0.93 (3H, s), 0.89 (9H, s), 0.45 (1H, dd,  $J$  = 8.6, 4.6 Hz), 0.10 (9H, s), 0.00 (3H, s), –0.01 (3H, s), –0.12 (1H, br t,  $J$  = 5.1 Hz).

**5.11. 5-[(1S,2R)-1-[(1S,3aR,4S,7aR)-4-(tert-Butyl-dimethyl-silanyloxy)-7a-methyl-octahydro-inden-1-yl]-2-(3-methyl-3-trimethylsilanyloxy-butyl)-cyclopropyl]-1,1,1-trifluoro-2-trifluoromethyl-pent-3-yn-2-ol (16)**

A two-necked 25 mL round-bottomed flask equipped with stir bar, Claisen adapter with rubber septum, and funnel (with cooling bath) was charged with **15** (480 mg, 0.95 mmol) and tetrahydrofuran (8 mL). The funnel was connected to container with hexafluoroacetone and cooled (acetone, dry ice). The reaction mixture was cooled to –70 °C and 1.6 M butyllithium in hexane (0.90 mL, 1.44 mmol) was added dropwise. After 30 min hexafluoroacetone was added (the container's valve was opened twice). The reaction mixture was stirred at –70 °C for 1 h then solution of saturated ammonium chloride (3 mL) was added. The mixture was dissolved by the addition of a solution of saturated ammonium chloride (50 mL) and extracted three times with hexane (50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated. The residue was chromatographed on column (200 mL) using 15:1 → 10:1 hexane/ethyl acetate as mobile phases to give 560 mg (88%) of **16** as colorless oil used directly in the next step.

**5.12. (1S,3aR,4S,7aR)-1-[(1S,2R)-2-(3-Hydroxy-3-methyl-butyl)-1-(5,5,5-trifluoro-4-hydroxy-4-trifluoromethyl-pent-2-ynyl)-cyclopropyl]-7a-methyl-octahydro-inden-4-ol (17)**

The mixture of **16** (560 mg, 0.84 mmol) and 1 M tetrabutylammonium fluoride in tetrahydrofuran (3 mL) was stirred at 70 °C for 24 h. A new portion of 1 M tetrabutylammonium fluoride in tetrahydrofuran (3 mL) was added, and the solution was stirred for next 24 h. Then the mixture was dissolved by the addition of ethyl acetate (100 mL) and washed five times with water (30 mL), brine (20 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and evaporated. The oil residue was chromatographed on column (100 mL) using 2:1 hexane/ethyl acetate as mobile phase. Mixture fractions were pooled, evaporated, and chromatographed on column (75 mL) using 1.5:1 hexane/ethyl acetate. Fractions containing product were pooled and evaporated to give 325 mg (80%) of **17** as colorless oil; H NMR (CDCl<sub>3</sub>): 4.07 (1H, br s), 2.73 (1H, d,  $J$  = 18.0 Hz), 2.36 (1H, d,  $J$  = 18.0 Hz), 2.09–2.00 (1H, m), 1.90–1.74 (3H, m), 1.70–1.11 (14H, m), 1.26 (6H, s), 1.03–0.83 (2H, m), 1.01 (3H, s), 0.55 (1H, dd,  $J$  = 9.3, 4.9 Hz), 0.14 (1H, br t,  $J$  = 5.5 Hz).

**5.13. (1S,3aR,7aR)-1-[(1S,2R)-2-(3-Hydroxy-3-methyl-butyl)-1-(5,5,5-trifluoro-4-hydroxy-4-trifluoromethyl-pent-2-ynyl)-cyclopropyl]-7a-methyl-octahydro-inden-4-one (18)**

To the suspension of pyridinium dichromate (807 mg, 2.15 mmol) and Celite (830 mg) in dichloromethane (10 mL) was added dropwise **17** (325 mg, 0.67 mmol)

in dichloromethane (2 mL). The mixture was stirred at room temperature for 4 h and additional portion of pyridinium dichromate (113 mg, 0.300 mmol) was added. After 1 h the reaction mixture was filtered through column with silica gel (25 mL) using 10:1  $\rightarrow$  5:1 dichloromethane/ethyl acetate as mobile phases. The fractions containing product were pooled and evaporated to give 245 mg (76%) of **18** as colorless oil; H NMR ( $\text{CDCl}_3$ )  $\delta$  2.75 (1H, d,  $J$  = 18.3 Hz), 2.41 (1H, dd,  $J$  = 11.4, 7.0 Hz), 2.33–2.16 (4H, m), 2.10–1.98 (2H, m), 1.96–1.85 (2H, m), 1.78–1.38 (9H, m), 1.28–1.22 (1H, m), 1.28 (3H, s), 1.26 (3H, s), 0.93–0.83 (1H, m), 0.72 (3H, s), 0.62 (1H, dd,  $J$  = 9.3, 4.9 Hz), 0.12 (1H, br t,  $J$  = 5.5 Hz).

#### 5.14. 1 $\alpha$ ,25-Dihydroxy-20S-(4-hydroxy-4-trifluoromethyl-5-trifluoro-2-pentynyl)-21,22R-cyclo-cholecalciferol (**19**)

A 1.6 M butyllithium in hexane (1.41 mL, 2.26 mmol) was added dropwise to the solution of **8** (1.32 mg, 2.26 mmol) in tetrahydrofuran (10 mL) at  $-78^\circ\text{C}$ . The flask was protected from light and the resulting deep red solution was stirred at  $-78^\circ\text{C}$  for 15 min then **18** (233 mg, 0.49 mmol) in tetrahydrofuran (1.6 mL) was added dropwise. The reaction mixture was stirred at this temperature for 4.5 h, removed from the bath, and then poured into ethyl acetate (50 mL) and brine (50 mL). The water fraction was extracted three times with ethyl acetate (50 mL), dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The oil residue was chromatographed on column (100 mL, protected from light) using 5:1 hexane/ethyl acetate as mobile phase. Fractions containing product were pooled and evaporated to give 280 mg of a colorless oil product, which was treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (4 mL). The reaction mixture was stirred at room temperature for 24 h. The mixture was dissolved by the addition of ethyl acetate (100 mL) and washed six times with water (30 mL), brine (50 mL), dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The oil residue was chromatographed on column (40 mL, protected from light) using 4:1 ethyl acetate/hexane as mobile phase. Fractions containing product were pooled and evaporated to give a colorless oil product, which was dissolved in methyl acetate (20 mL) and evaporated (4 times) to give 170 mg (85%) of **19** as white foam;  $[\alpha]_D^{30}$   $-34.0$  ( $c$  0.42,  $\text{CHCl}_3$ ), UV  $\lambda_{\text{max}}$  (EtOH): 213 nm ( $\epsilon$  14384), 264 nm ( $\epsilon$  15952). H NMR ( $\text{CDCl}_3$ )  $\delta$  6.37 (1H, d,  $J$  = 11.1 Hz), 5.99 (1H, d,  $J$  = 11.3 Hz), 5.33 (1H, s), 4.99 (1H, s), 4.43 (1H, br s), 4.23 (1H, br s), 2.84–2.81 (1H, m), 2.78 (1H, d,  $J$  = 18.1 Hz), 2.62–2.58 (1H, m), 2.31 (1H, dd,  $J$  = 13.5, 6.5 Hz), 2.25 (1H, d,  $J$  = 18.1 Hz), 2.06–1.84 (5H, m), 1.77–1.38 (13H, m), 1.32–1.25 (3H, m), 1.27 (3H, s), 1.25 (3H, s), 0.88–0.77 (1H, m), 0.63 (3H, s), 0.63–0.58 (1H, m), 0.11–0.09 (1H, m), HR-MS calcd for  $\text{C}_{33}\text{H}_{44}\text{F}_6\text{O}_4$   $[\text{M}+\text{Na}]^+$  641.3036; found 641.3042.

#### 5.15. 1 $\alpha$ ,25-Dihydroxy-20S-(4-hydroxy-4-trifluoromethyl-5-trifluoro-2-pentynyl)-21,22R-cyclo-19-nor-cholecalciferol (**20**)

Following the procedure for the synthesis of **19**, a 1.6 M butyllithium in hexane (0.66 mL, 1.06 mmol)

was added to solution of **9** (603 mg, 1.06 mmol) in anhydrous tetrahydrofuran (6 mL) and then **18** (85 mg, 0.18 mmol) in tetrahydrofuran (1.6 mL) was added dropwise. After chromatography the oil residue was treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (3 mL). The reaction mixture was stirred at room temperature for 8 h and a new portion of 1 M tetrabutylammonium fluoride in tetrahydrofuran (1.5 mL) was added. The reaction mixture was stirred for next 16 h. Workup and chromatography (ethyl acetate as mobile phase) gave 91 mg (94%) of **20** as white foam,  $[\alpha]_D^{31}$   $-2.32$  ( $c$  0.43,  $\text{CHCl}_3$ ), UV  $\lambda_{\text{max}}$  (EtOH): 243 nm ( $\epsilon$  30242), 251 nm ( $\epsilon$  35639), 261 nm ( $\epsilon$  24175). H NMR ( $\text{CDCl}_3$ )  $\delta$  6.30 (1H, d,  $J$  = 11.5 Hz), 5.38 (1H, d,  $J$  = 11.1 Hz), 4.14–4.10 (1H, m), 4.08–4.02 (1H, m), 2.79 (1H, d,  $J$  = 18.1 Hz), 2.79–2.70 (2H, m), 2.50–2.46 (1H, m), 2.25 (1H, d,  $J$  = 18.1 Hz), 2.27–2.18 (2H, m), 2.06–1.39 (18H, m), 1.32–1.21 (3H, m), 1.27 (3H, s), 1.25 (3H, s), 0.88–0.79 (1H, m), 0.63 (3H, s), 0.63–0.59 (1H, m), 0.10 (1H, dd,  $J$  = 5.3, 5.3 Hz), HR-MS calcd for:  $\text{C}_{32}\text{H}_{44}\text{F}_6\text{O}_4$   $[\text{M}+\text{Na}]^+$  607.3217; found 607.3226.

#### 5.16. Cell cultures

HL60 cells, derived from a patient with promyeloblastic leukemia,<sup>24</sup> were obtained for these studies from the ATCC. HL60-G<sup>33</sup> and HL60-40AF cells<sup>27</sup> are differentiation sensitive and resistant subclones of HL60 cells, respectively. These cells were propagated as a suspension culture in RPMI 1640 medium supplemented with 10% bovine calf serum (Hyclone, Logan, UT). The cells were kept at 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The cell number and viability were determined by hemocytometer counts and Trypan blue (0.2%) exclusion assay. Routine microbiology testing for mycoplasma was conducted. For all experiments the cells were suspended in fresh medium containing 1,25D or the equivalent volume of ethanol as a vehicle control. Each experiment was repeated at least three times.

#### 5.17. Chemicals and antibodies

1,25D and all analogs utilized in this study were synthesized by Bioxell, Inc. (Nutley, NJ) and were provided in 1 mg aliquots. Stock concentrations ( $2.5 \times 10^{-4}$  M) were prepared by dissolving analogs in filtered EtOH. MY4-RD1 and MO1-FITC antibody recognizing CD14 and CD11b, respectively, and isotype controls were purchased from Beckman-Coulter (Fullerton, CA). Propidium iodide was obtained from Sigma (St. Louis, MO). Primers for CYP24 and Acidic Riboprotein Subunit 0 (ARP0) were synthesized by the Molecular Resource Facility of UMDNJ (Newark, NJ). RNase-free DNase was obtained from Qiagen (Valencia, CA).

#### 5.18. Determination of markers of differentiation

Aliquots of  $1 \times 10^6$  cells were washed twice with 1 $\times$  phosphate-buffered saline (PBS), then incubated with 0.3  $\mu\text{L}$  MY4-RD1 and 0.3  $\mu\text{L}$  MO1-FITC on ice for

45 min to analyze the expression of differentiation markers. After the incubation the cells were washed three times with PBS and suspended in 1 mL PBS. Samples were analyzed using EPICS XL flow cytometer (Beckman–Coulter). The acquisition parameters were set for isotype controls. Data analysis was performed with EPICS XL software (Beckman–Coulter).

### 5.19. Cell cycle analysis

To determine cell cycle distributions, aliquots of  $1 \times 10^6$  cells were washed twice with PBS and fixed in 75% ethanol at  $-20^\circ\text{C}$  overnight, then washed twice and incubated with 100 U/mL of RNase A at  $37^\circ\text{C}$  for 1 h. The cell pellet was washed twice and resuspended in a propidium iodide solution (10  $\mu\text{g/mL}$ ). The cells were analyzed using an EPICS XL flow cytometer (Beckman–Coulter) and the cell cycle distribution was determined by Multicycle Software Program (Phoenix Flow System, San Diego, CA).

### 5.20. Quantitative RT-PCR

Aliquots of  $5 \times 10^6$  cells were harvested and washed twice with ice-cold 1×PBS. Total cellular RNA was extracted with RNeasy mini kit (Qiagen) and treated with RNase-free DNase according to manufacturer's protocol. RNA quality and final concentration was determined by spectrophotometric reading at 260 and 280 nm, and 1  $\mu\text{g}$  total RNA was reverse transcribed with MuLV RT primed with oligo-d(T) following manufacturer's protocol (Applied Biosystems, Foster City, CA). Relative quantification of target cDNA was performed on a Roche Lightcycler instrument with Faststart DNA Master<sup>plus</sup> Syber Green I kit (Hoffmann La Roche, Nutley, NJ) and gene specific primers. Primers were as follows: (1) CYP24: forward 5'-CAAACCGTGGAAGGCCTATC-3' and reverse 5'-AGTCTTCCCCTTCCAGGATCA-3' (70 bp), (2) ARP0: forward 5'-AGATGCAGCAGATCCGCAT-3' and reverse 5'-GTGGTGATACCTAAA GCCTG-3' (318 bp).<sup>34</sup>

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