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# Structure-Specific Control of Differentiation and Apoptosis of Human Promyelocytic Leukemia (HL-60) Cells by A-ring Diastereomers of 2-Methyl-1α,25-dihydroxyvitamin $D_3$ and Its 20-Epimer

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**ABSTRACT.** 1α,25-Dihydroxyvitamin $D_3$ (1α,25$(OH)_2D_3$) has been shown to modulate not only proliferation and differentiation but also apoptosis of malignant cells, indicating that it would be useful for the treatment of hyperproliferative diseases such as cancer and psoriasis. Little information is available concerning structural motifs of the 1α,25$(OH)_2D_3$ molecule responsible for modulation of differentiation and apoptosis. We synthesized all possible A-ring diastereomers of the 2-methyl-1α,25$(OH)_2D_3$ and its 20-epimer and evaluated their biological activities in human promyelocytic leukemia (HL-60) cells. Surprisingly, the potent analogues could be clearly divided into two groups: (i) those bearing the 1α- and 3β-hydroxyl groups on the A-ring were potent inducers of differentiation and growth inhibitors of HL-60 cells and (ii) those bearing the 1β-hydroxyl group together with either 3α- or 3β-hydroxyl groups on the A-ring were potent stimulators of apoptosis in these cells. We have clearly identified for the first time the structural motifs on the basis of the stereochemistry of both hydroxyl groups at positions 1 and 3 of the A-ring of the 1α,25$(OH)_2D_3$ molecule responsible for the induction of differentiation and apoptosis of HL-60 cells. These findings provide useful information not only for structure–function studies of 1α,25$(OH)_2D_3$ analogues but also for the development of therapeutic agents for the treatment of leukemia and other cancers. BIOCHEM PHARMACOL **60**;12:1937–1947, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** apoptosis; A-ring stereoisomers; differentiation; growth inhibition; HL-60 cells; 2-methyl-1α,25$(OH)_2D_3$; 2-methyl-20-epi-1α,25$(OH)_2D_3$; structural motif

The biologically active form of vitamin $D_3$, 1α,25-dihydroxyvitamin $D_3$ [1α,25$(OH)_2D_3$]§ [1], is a potent regulator of calcium and phosphate metabolism in the intestine, kidney, and bone [2, 3]. It has also been found to modulate growth and differentiation of a variety of normal and malignant cells. Due to its highly pluripotent effects, many efforts have been made to dissociate the partial effects of 1α,25$(OH)_2D_3$ by modification of its structure and to generate potential therapeutic agents for the treatment of osteoporosis, cancer, and psoriasis [4–7].

The action of 1α,25$(OH)_2D_3$ is mediated via its binding to the VDR [8], a member of the nuclear receptor superfamily [9]. The VDR forms a heterodimer with another member of this family, the RXR [10], and regulates gene expression via binding to specific DNA sequences, the VDRE [11]. Thus, an ideal analogue of 1α,25$(OH)_2D_3$ will have a high binding affinity for VDR, thus forming a stable VDR/RXR complex, and bind strongly to VDRE. Extensive structure–function studies of the vitamin D molecule [12] have shown that beside the 25-hydroxyl group of the side chain, the 1α- and 3β-hydroxyl groups of the A-ring are also required for effective binding to the VDR and thus for expression of its full biological activities. Indeed, a previous study showed that A-ring diastereomers of 1α,25$(OH)_2D_3$ displayed diminished genomic responses due to lack of affinity for VDR [13]. Recently, we synthesized and evaluated the biological activities of all the possible A-ring diastereomers of 2-methyl-1α,25$(OH)_2D_3$ and its 20-epimer [14–16]. Our studies revealed that the 2α-methyl-20-epi-1α,25$(OH)_2D_3$ was approximately 200-fold more transcriptionally active than 1α,25$(OH)_2D_3$. Surprisingly, 2β-methyl-20-epi-3-epi-1β,25$(OH)_2D_3$, which possesses stereochemically inverted hydroxyl groups at positions 1 and 3 of the natural hormone together with the 20-epi side chain, was approximately 4-fold more transcriptionally

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§ *Abbreviations:* 2-methyl-1α,25$(OH)_2D_3$, 2-methyl-1α,25-dihydroxyvitamin $D_3$; 2-methyl-20-epi-1α,25$(OH)_2D_3$, 2-methyl-20-epi-1α,25-dihydroxyvitamin $D_3$; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin $D_3$; VDR, vitamin D receptor; RXR, retinoid X receptor; and VDRE, vitamin D-responsive element.

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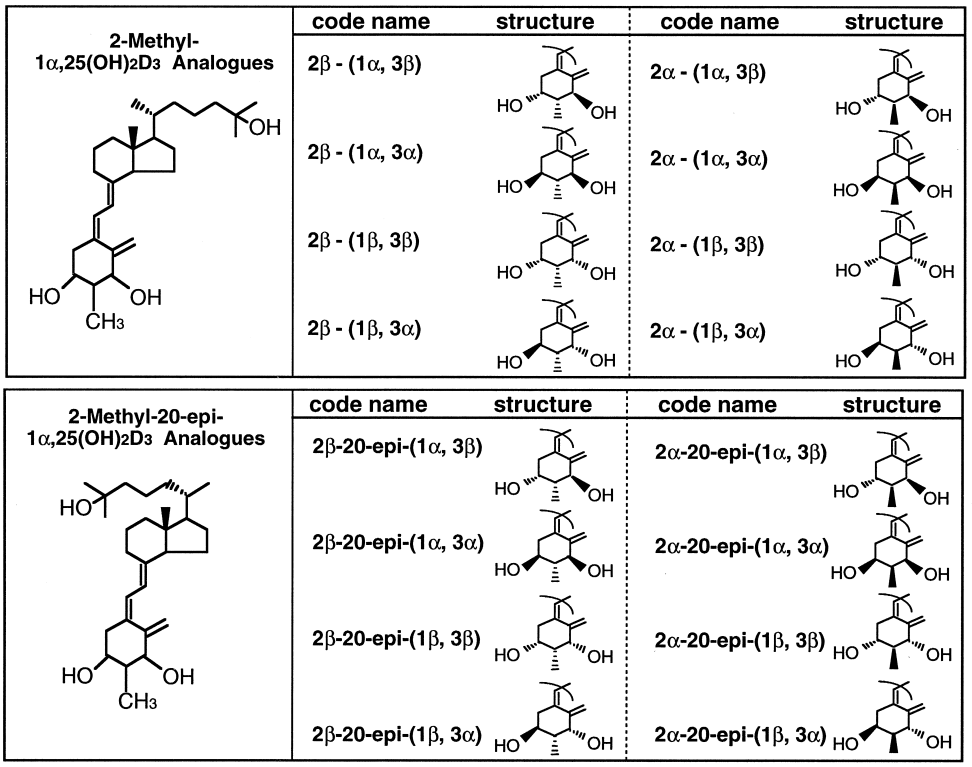


**FIG. 1. Chemical structures and code names assigned to all possible A-ring diastereomers of 2-methyl-1α,25(OH)$_2$D$_3$ and its 20-epimers.**

active than 1α,25(OH)$_2$D$_3$ [16]. This implied that either the α- or β-orientations of the hydroxyl groups at positions 1 and 3 of the A-ring are not necessarily required for full expression of vitamin D action. It is plausible that the conformational changes in the A-ring itself induced by alterations of the stereochemistry of both hydroxyl groups in positions 1 and 3 of the A-ring along with the 2-methyl substitution in the A-ring may be responsible for the above unique biological activity. Therefore, characterization of the possible roles of both hydroxyl groups at positions 1 and 3 and 2-methyl substituents of the A-ring might lead to the development of new agents useful for the treatment of vitamin D-related diseases. The present study was undertaken to further clarify the biological activities of the 2-methyl-1α,25(OH)$_2$D$_3$ and its 20-epimer in terms of their effects on cell cycle phase distribution, cell surface CD11b antigen expression, and apoptosis of HL-60 cells.

## MATERIALS AND METHODS

### *Chemicals*

1α,25(OH)$_2$D$_3$ was purchased from Solvey-Duphar Co. All possible A-ring diastereomers of 2-methyl-1,α,25(OH)$_2$D$_3$ and its 20-epimer were synthesized by Konno *et al.* [14] and Fujishima *et al.* [15]. On the basis of the configuration of a methyl group at position 1 of the A-ring, the 2-methyl- and 2-methyl-20-epi A-ring diastereomers were classified into two groups, namely 2β- or 2α-methyl and 2β- or 2α-methyl-20-epi A-ring diastereomers. All sixteen analogues [16] tested are shown in Fig. 1. The analogues were dissolved in absolute ethanol as stock solutions at $10^{-4}$ M and stored at −35° protected from light. All other reagents were of the highest analytical grade commercially available.

### *HL-60 Cells and Synchronization of Cell Cycle at S Phase by an Excess of Thymidine*

HL-60 cells were obtained from Dr. Y. Seino of the Okayama University School of Medicine. The cells were maintained in continuous culture in RPMI-1640 medium (Nissui Seiyaku Co., Ltd.) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (GIBCO BRL), kanamycin (0.06 mg/mL) (Sigma) at 37° in a humidified atmosphere of 5% $CO_2$ in air. The doubling time of HL-60 cells was approximately 24 hr. For synchronization at S phase, cells ($4 \times 10^6$) were cultured in 30 mL of RPMI-1640 medium for 24 hr and subsequently cultured for 16 hr in RPMI-1640 medium supplemented with 2.5 mM thymidine. After washing with Ca, Mg-free phosphate-buffered saline [PBS(−)] twice, the cells were cultured in normal medium for 10 hr and then in 2.5 mM thymidine medium for 16 hr. The cells obtained were used in the following experiments.

### *Flow Cytometry*

Cells ($10^5$ cells/well) were plated in 24-well tissue culture plates and cultured for 3 days with 1α,25(OH)$_2$D$_3$ or the

analogues ($10^{-8}$ M) in RPMI-1640 medium at 37° in a humidified atmosphere of 5% $CO_2$ in air. To reduce the effects of contact inhibition, control cells were adjusted to reach 60–70% confluency at the time of FACS analysis. Each group of cells was collected and washed once with PBS(−). Then, the cells were resuspended in PBS(−) containing 0.2% Triton-X and 100 μg RNase and incubated at 37° for 1 hr. Cells were washed with PBS(−) and incubated with 0.5 mL of DNA-staining solution containing propidium iodide (50 μg/mL) at 4° for 20 min. The cells were analyzed with a flow cytometer equipped with an argon laser (488 nm, Beckton Dickinson FACScan™) and cell cycle distribution was analyzed using ModiFiT LT(Verity).

### *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 1α,25$(OH)_2D_3$ or the analogues ($10^{-8}$ M) as described above for the flow cytometry. Each group of cells was then collected and washed once with PBS(−). Then, the cells ($2 \times 10^5$) were resuspended in 100 μL of diluent solution containing 1% BSA and 1% sodium azide and incubated with 10 μL of human monoclonal fluorescein isothiocyanate (FITC)-conjugated CD11b antibody (Sigma) in the dark for 30 min at room temperature. The cells were washed once with diluent solution and then fixed in 300 μL of PBS(−) containing 2% paraformaldehyde. Fluorescence was detected on a Beckton Dickinson FACScan™ at an 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 counted per treatment.

### *Nuclear Staining Assay*

Cells ($10^5$ cells/well) were placed in 24-well tissue culture plates and cultured for 5 days in RPMI-1640 medium with 1α,25$(OH)_2D_3$ or the analogues ($10^{-8}$ M) under the same conditions as described above. Each group of cells was then collected and washed once with PBS(−). The cells were fixed with 1% glutanaldehyde in PBS(−) for 30 min and then washed with PBS(−). The preparations were suspended in PBS(−) and stained with 1 mM Hoechst 33258 (Calbiochem) in PBS(−) for 10 min. The cells were viewed under an OLYMPUS IX70 microscope. Condensed and fragmented nuclei and percentages were calculated by counting. Three random fields of view were observed with a minimum number of 200 cells scored in each 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 5 days in RPMI-1640 medium with 1α,25$(OH)_2D_3$ or the analogues ($10^{-8}$ M) under the same conditions as described 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 with an *in situ* apoptosis detection kit (Takara Shuzo Co., Ltd.) 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 and examined for fragmentation. DNA was electrophoresed in 2% agarose gels, which were stained with ethidium bromide and observed under ultraviolet light [17, 18].

### *Caspase-3 Activity Assay (Fluorogenic Substrate Assay)*

The activities of caspase-3 were measured by the fluorometric assay according to the manufacturer's instructions. Briefly, untreated or vitamin D compound-treated cells were incubated for 5 days and then harvested. Cells ($10^6$) were lysed in 0.1 M HEPES buffer, pH 7.4, containing 2 mM dithiothreitol, 0.1% 3-[(3-cholamidoldimethylammonio]-1-propanesulfonic acid (Chaps), and 1% sucrose. Cell lysates were incubated with 20 μL of an 80-mM solution of the fluorogenic substrate, Ac-DMQD-MCA (Ac-Asp-Met-Gln-Asp-MCA, Peptide Institute, Inc.), which is preferentially cleaved by caspase-3, for 60 min at 37°, and the release of 7-amino-4-methylcoumarin (AMC) was measured with excitation at 400 nm and emission at 505 nm using a fluorescence spectrophotometer (Bio-Rad Laboratories, Fluoromark™). The amount of protein in each cell extract was measured using a protein assay kit (Bio-Rad) [19].

### *Statistics*

The results are expressed as means ± SEM. Paired or unpaired Student's *t*-test was used to determine the significance of difference, with a value of $P < 0.05$ being considered statistically significant.

## RESULTS

### *Effect on Cell Cycle Phase Distribution*

1α,25$(OH)_2D_3$ has been shown to promote cell cycle arrest in G1 phase in malignant cells, including leukemic cells and breast cancer cells [20, 21]. To compare the antiproliferative effect on HL-60 cell growth, we measured cell cycle phase distribution of HL-60 cells treated with 1α,25$(OH)_2D_3$ or the analogues. Results from three indi-

**TABLE 1. Cell cycle distribution in HL-60 cells treated with 2-methyl-1α,25(OH)$_2$D$_3$ analogues**

| D analogues | G0/G1 (%) | S (%) | G2/M (%) |
|---|---|---|---|
| Control | 54.5 ± 3.3 | 37.1 ± 3.3 | 8.5 ± 0.2 |
| 1α,25(OH)$_2$D$_3$ | 82.6 ± 0.5** | 8.4 ± 0.8†† | 9.1 ± 0.2 |
| 2β - (1α, 3 β) | 69.1 ± 3.2* | 22.6 ± 2.5† | 8.3 ± 0.7 |
| 2β - (1α, 3 α) | 56.8 ± 2.5 | 35.0 ± 2.0 | 8.2 ± 0.6 |
| 2β - (1β, 3 β) | 56.9 ± 1.5 | 34.9 ± 1.4 | 8.3 ± 0.4 |
| 2β - (1β, 3 α) | 57.6 ± 3.1 | 35.6 ± 3.1 | 6.8 ± 0.4‡ |
| 2α - (1α, 3 β) | 83.8 ± 1.0** | 6.3 ± 0.6† | 9.9 ± 1.5 |
| 2α - (1α, 3 α) | 63.0 ± 2.1 | 29.1 ± 2.6 | 7.9 ± 0.5 |
| 2α - (1β, 3 β) | 57.6 ± 2.6 | 35.2 ± 2.2 | 7.2 ± 0.4‡ |
| 2α - (1β, 3 α) | 57.1 ± 3.1 | 35.6 ± 2.5 | 7.4 ± 0.8 |

Experiments were repeated three times. Results are means ± SEM.

Values sharing the same character are significantly different from the respective control (*, †, ‡ $P < 0.05$, **, ††, ‡‡ $P < 0.01$, ***, †††, ‡‡‡ $P < 0.001$).

vidual experiments after incubation for 3 days using flow cytometry of propidium iodide-stained cells are depicted in Table 1. The percentages of HL-60 cells in the G0/G1 phase were significantly ($P < 0.01$) increased from 54.5% (vehicle-treated cells) to 82.6% (1α,25(OH)$_2$D$_3$-treated), while those in S phase were significantly ($P < 0.05$) decreased from 37% (control) to 8% (1α,25(OH)$_2$D$_3$-treated). Similar to 1α,25(OH)$_2$D$_3$, the 2-methyl-1α,25(OH)$_2$D$_3$ analogues, 2β-(1α,3β) and 2α-(1α,3β), caused HL-60 cells to accumulate in the G0/G1 phase with a decrease in S phase of the cell cycle. Among the 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues, the 2β-20-epi-(1α,3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3β), and 2α-20-epi-(1α,3α) caused HL-60 cells to accumulate in the G0/G1 phase with a decrease in S phase of the cell cycle (Table 2). Thus, the HL-60 cell growth inhibition exhibited by 1α,25(OH)$_2$D$_3$ and its analogues is likely due to their inhibition of cellular progression to G0/G1 + S phases via a VDR-mediated mechanism.

### *Effect on the Expression of CD11b Antigen and Appearance of Apoptotic Cells (Nuclear Staining)*

Expression of cell surface CD11b antigen is one of the major differentiation markers of HL-60 cells to monocytes/macrophages [22]. The HL-60 cells were cultured for 3 days with either 1,α,25(OH)$_2$D$_3$ or the analogues and the expression of CD11b was tested. It can be seen from Fig. 2 (panel A) that 1α,25(OH)$_2$D$_3$ and the analogues 2β-(1α,3β), 2α-(1α,3β), and 2α-(1α,3α) induced expression of CD11b antigen on the surfaces of HL-60 cells about 10-, 3-, 11-, and 2-fold, respectively, greater than the control. These results clearly indicate that the analogues 2β-(1α,3β), 2α-(1α,3β), and 2α-(1α,3α) cause differentiation of HL-60 cells. The CD11b antigen expression was increased in a dose- and time-dependent manner in the presence of 1α,25(OH)$_2$D$_3$ and the aforementioned three analogues (data not shown). However, the other analogues did not induce expression of CD11b antigen on the surfaces of HL-60 cells at any time point or dose examined. Furthermore, the HL-60 cells treated with 1α,25(OH)$_2$D$_3$, 2β-(1α,3β), 2α-(1α,3β), and 2α-(1α,3α) became longer and thinner, giving a macrophage-like appearance (data not shown).

To clarify whether the inhibitory effect of 1α,25(OH)$_2$D$_3$ and its analogues on HL-60 cell growth is correlated with their effects on induction of apoptosis, the HL-60 cells were cultured for 5 days with either 1α,25(OH)$_2$D$_3$ or the analogues and the number of apoptotic cells was counted. The apoptotic cells were calculated as the percentage of 200 cells scored in random fluoromicroscopic fields of view. Figure 2 (panel B) shows that 2β-(1α,3α), 2β-(1β,3β), 2β-(1β,3α), 2α-(1β,3β) and

**TABLE 2. Cell cycle distribution in HL-60 cells treated with 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues**

| D analogues | G0/G1 (%) | S (%) | G2/M (%) |
|---|---|---|---|
| Control | 54.5 ± 3.3 | 37.1 ± 3.3 | 8.5 ± 0.2 |
| 1α,25(OH)$_2$D$_3$ | 82.6 ± 0.5** | 8.4 ± 0.8†† | 9.1 ± 0.2 |
| 2β-20-epi-(1α, 3β) | 88.2 ± 0.4*** | 4.0 ± 0.4††† | 7.7 ± 0.1‡ |
| 2β-20-epi-(1α, 3α) | 55.2 ± 1.2 | 36.5 ± 0.9 | 8.3 ± 0.3 |
| 2β-20-epi-(1β, 3β) | 62.1 ± 6.8 | 32.6 ± 4.9 | 5.3 ± 1.8 |
| 2β-20-epi-(1β, 3α) | 87.6 ± 0.6*** | 7.6 ± 2.1†† | 4.9 ± 2.5 |
| 2α-20-epi-(1α, 3β) | 90.3 ± 2.2*** | 4.1 ± 0.3††† | 5.6 ± 2.4 |
| 2α-20-epi-(1α, 3α) | 86.7 ± 2.9** | 6.6 ± 0.2††† | 6.8 ± 3.0 |
| 2α-20-epi-(1β, 3β) | 60.9 ± 4.3 | 34.0 ± 3.5 | 5.2 ± 0.8‡‡ |
| 2α-20-epi-(1β, 3α) | 60.8 ± 6.5 | 33.6 ± 5.2 | 5.6 ± 1.3 |

Experiments were repeated three times. Results are means ± SEM.

Values sharing the same character are significantly different from the respective control (*, †, ‡ $P < 0.05$, **, ††, ‡‡ $P < 0.01$, ***, †††, ‡‡‡ $P < 0.001$).

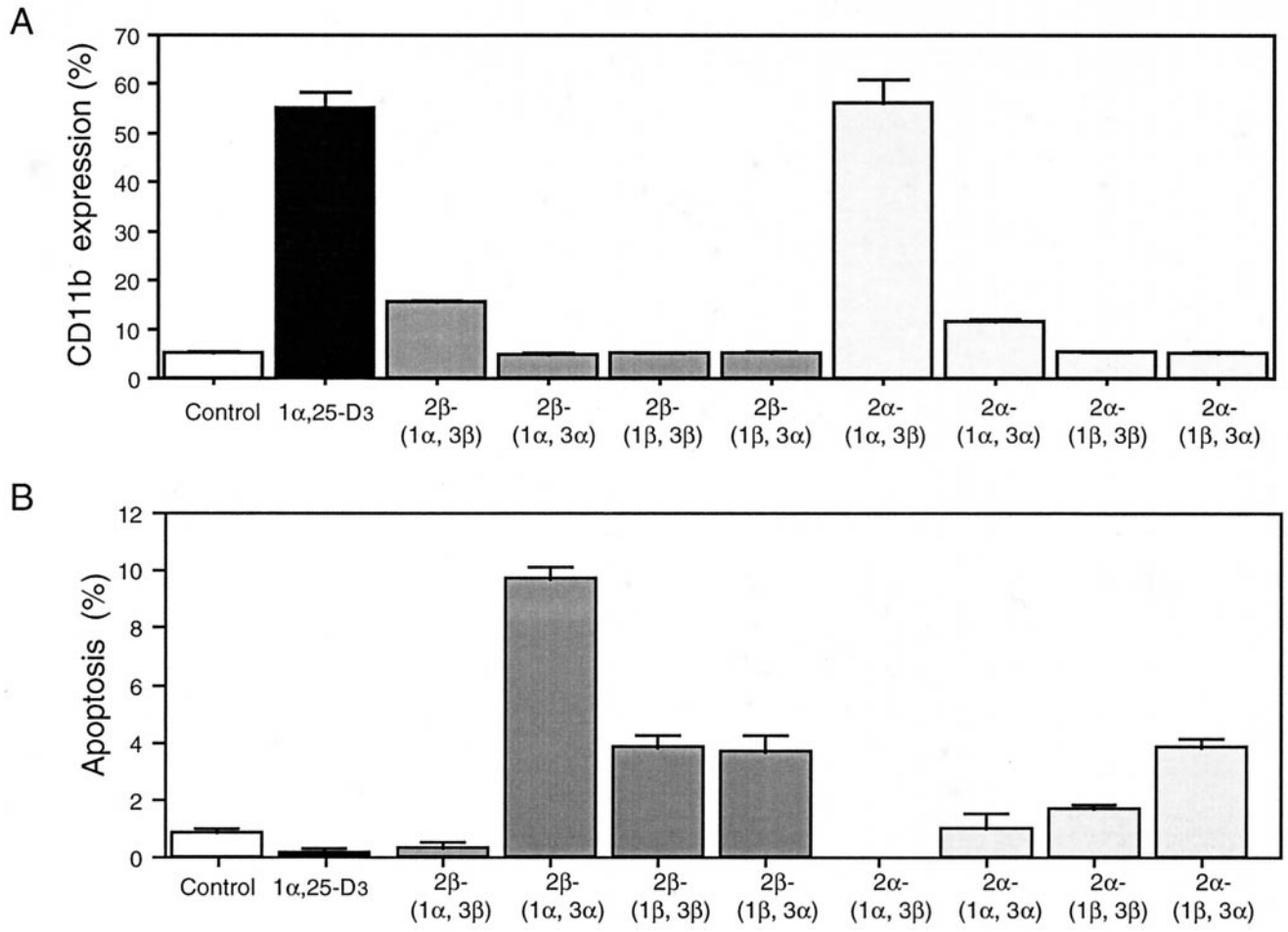


**FIG. 2. Cell surface CD11b antigen-positive cell number (panel A) and apoptotic cell number (panel B) in culture of HL-60 cells treated with vehicle, 1α,25(OH)$_2$D$_3$, or its 2-methyl analogues ($10^{-8}$ M) for 3 days. The cells were analyzed by FACS for the expression of CD11b antigen. In duplicate cultures, changes in nuclear morphology that were characteristic of apoptosis were observed by staining with the DNA-specific fluorochrome bisbenzimide (Hoechst 33258). Data are expressed as percentage of apoptotic cells. Cells with condensed chromatin and three or more fragmented nuclei were considered to be apoptotic. A minimum of 200 cells were scored in each field. Each value represents the mean ± SEM of two independent determinations from three separate experiments.**

2α-(1β,3α), but not 1α,25(OH)$_2$D$_3$, caused apoptosis of HL-60 cells. It has been reported that 1α,25(OH)$_2$D$_3$ itself has no apoptosis-stimulating effect on HL-60 cells, but it has an inhibitory effect on drug-induced apoptosis of HL-60 cells [17, 23]. Our results are consistent with those reported previously. Surprisingly, the above five analogues all have stereochemically inverted orientations of the hydroxyl groups at positions 1 and 3 (1β and 3α) of the A-ring.

Similar results were observed in the 20-epi analogues. From Fig. 3 (panel A), it can be seen that 2β-20-epi-(1α, 3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3β) and 2β-20-epi-(1α, 3α) induced expression of CD11b antigen on the cell surfaces of HL-60 cells 9-, 11-, 12-, and 11-fold, respectively, greater than the control. On the other hand, the three 20-epi analogues, 2β-20-epi-(1α,3α), 2β-20-epi-(1β,3β), and 2α-20-epi-(1β,3α), induced apoptosis of HL-60 cells (Fig. 3, panel B). These results clearly indicated that there is an inverse relationship between the induction of differentiation and apoptosis of HL-60 cells.

### *Effect on the Appearance of DNA-Fragmented Cells (TUNEL Staining)*

Figure 4 shows the fluoromicroscopic pictures of the HL-60 cells treated with either 1α,25(OH)$_2$D$_3$ or vehicle using a TUNEL method [24]. No apoptotic cells were observed in the vehicle or 1α,25(OH)$_2$D$_3$ treated HL-60 cells. On the other hand, the 2-methyl analogues bearing the 1β- or 3α-hydroxyl groups on the A-ring exhibited significant ability to cause apoptosis (Fig. 5). Similarly, the 2-methyl-20-epi analogues bearing the 1β- or 3α-hydroxyl groups on the A-ring exhibited significant ability to cause apoptosis (Fig. 6).

### *Effect on DNA Fragmentation (Agarose Gel Electrophoresis)*

Figure 7 shows that the 2-methyl analogues bearing the 1β- or 3α-hydroxyl groups on the A-ring (lanes 4–6, 8–10) but not 1α,25(OH)$_2$D$_3$ (lane 2), induced DNA fragmentation.

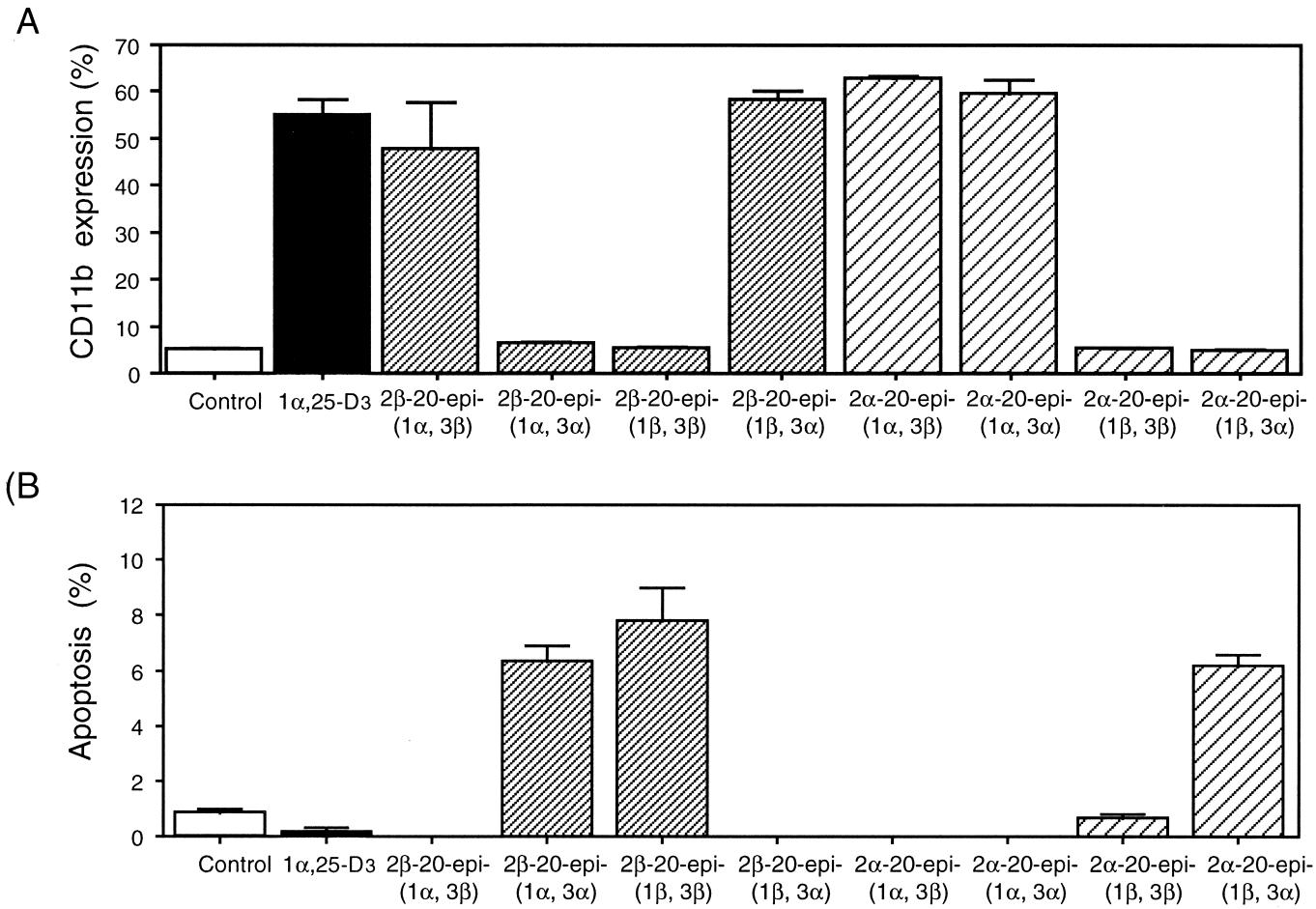


**FIG. 3. Cell surface CD11b antigen-positive cell number (panel A) and apoptotic cell number (panel B) in culture of HL-60 cells treated with vehicle, 1α,25(OH)$_2$D$_3$ or its 2-methyl-20-epimers ($10^{-8}$ M) for 3 days. The details of cell surface antigen expression analysis and nuclear staining assay are described in the legend to Fig. 2. Each value represents the mean ± SEM of two independent determinations from three separate experiments.**

Their DNA ladders were prominent when cells were treated for 5 days with the analogues. Among the 2-methyl-20-epi analogues, 2β-20-epi-(1α,3α) and 2β-20-epi-(1β,3β) (lanes 12 and 13) induced DNA fragmentation (Fig. 7). The DNA fragmentation induced by the analogues 2β-(1α,3α), 2β-(1β,3β), 2β-(1β,3α), 2α-(1α,3α), 2α-(1β,3β), and 2α-(1β,3α) (lanes 4–6, 8–10) was more prominent than that induced by 2β-20-epi-(1α,3α) (lane 12) and 2β-20-epi-(1β,3β) (lane 13), suggesting that the 2-methyl analogues with a natural side chain had a stronger apoptosis-inducing effect than the 20-epi counterparts in the HL-60 cells.

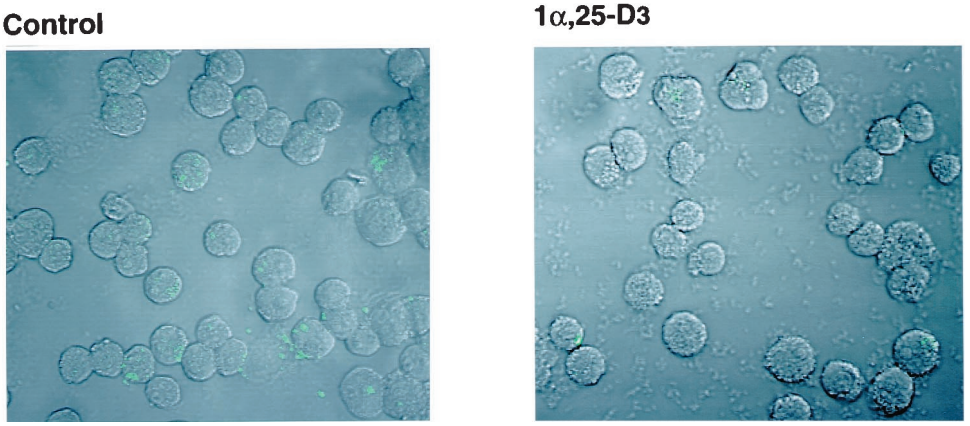


**FIG. 4. Effects of 1α,25(OH)$_2$D$_3$ on the appearance of DNA-fragmented cells. The HL-60 cells were treated for 5 days with the vehicle or 1α,25(OH)$_2$D$_3$ ($10^{-8}$ M) and stained with the TUNEL method.**

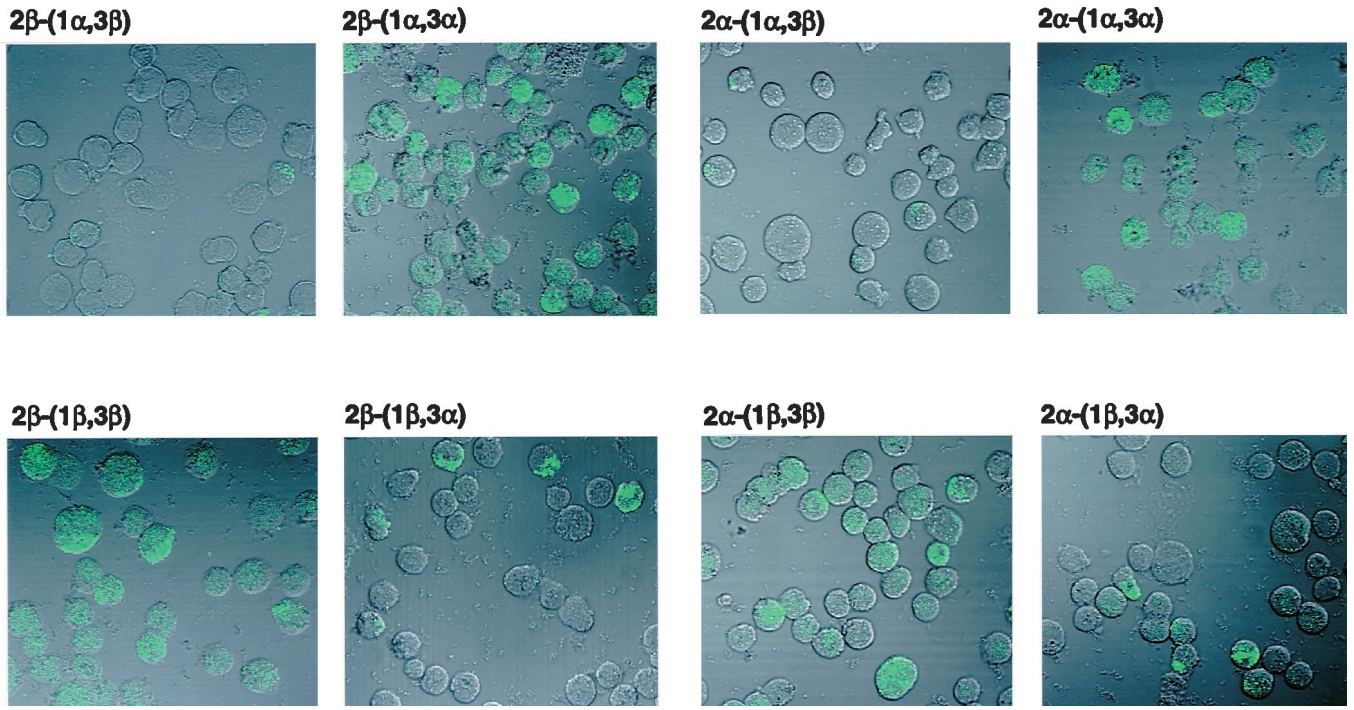


**FIG. 5. Effects of 2-methyl-1α,25(OH)$_2$D$_3$ analogues on the appearance of DNA-fragmented cells. The HL-60 cells were treated for 5 days with the 2-methyl-1α,25(OH)$_2$D$_3$ analogues ($10^{-8}$ M) and stained with the TUNEL method. The nuclei were visualized by fluorescence microscopy.**

### *Effect on Caspase-3 Activity*

Figure 8 shows that in the 2-methyl analogues, 2β-(1α,3α), 2β-(1β,3β), and 2α-(1β,3α) significantly ($P < 0.05$) increased caspase-3 activity, but 1α,25(OH)$_2$D$_3$ did not induce any changes in caspase-3 activity compared to the control cells. The analogue 2α-(1α,3β) significantly decreased caspase-3 activity ($P < 0.05$). Of the 20-epi analogues, 2β-20-epi-(1β,3β) and 2α-20-epi-(1β,3α) significantly increased ($P < 0.05$) caspase-3 activity. Conversely, the analogues, 2β-20-epi-(1α,3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3β), and 2α-20-epi-(1α,3α) significantly decreased caspase-3 activity.

## DISCUSSION

In this study, we examined the regulatory activities of all A-ring diastereomers of 2-methyl and 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues on the proliferation, differentiation, and apoptosis of HL-60 cells. Our results indicated that the 2-methyl-1α,25(OH)$_2$D$_3$ analogues bearing the 1α- and 3β-hydroxyl groups and the 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues bearing the 1α-hydroxyl group together with the 3α- or 3β-hydroxyl groups on the A-ring induced differentiation and inhibited proliferation of HL-60 cells, but failed to induce apoptosis of these cells. Previously, we reported that the aforementioned analogues were more potent than 1α,25(OH)$_2$D$_3$ in terms of binding affinity for VDR and transactivation on rat 25-hydroxyvitamin D$_3$-24-hydroxylase gene promoter activity and human osteocalcin gene promoter activity in transfected MG-63 cells [16]. From these results, it is obvious that these potent analogues exert their vitamin D actions via a VDR-dependent signaling pathway in HL-60 cells. The analogue 2β-20-epi-(1β,3α) is an interesting exception among the analogues tested. Despite the lack of both 1α- and 3β-hydroxyl groups on the A-ring, it had approximately 4-fold greater transcriptional activity on human osteocalcin gene promoter [16] compared to 1α,25(OH)$_2$D$_3$ and comparable abilities to 1α,25(OH)$_2$D$_3$ in inducing G1 arrest and differentiation of HL-60 cells into monocytes/macrophages *in vitro*. Since 2β-20-epi-(1β,3α) differed markedly from 1α,25(OH)$_2$D$_3$ in the conformational distribution of the side chain and the A-ring, its *in vitro* binding affinity for VDR was only 7% compared to 1α,25(OH)$_2$D$_3$ (100%) [15]. Previously, we reported that 2β-20-epi-(1β,3α) could bind VDR with comparable ability to 1α,25(OH)$_2$D$_3$ and that it forms a heterodimer with RXRα more efficiently than 1α,25(OH)$_2$D$_3$ in VDR-GAL4 and RXRα-GAL4 luciferase reporter assay systems in transfected cells [16]. At present, the molecular mechanisms responsible for the high biological activities of 2β-20-epi-(1β,3α) are not clearly understood and are currently under investigation in our laboratory.

1α,25(OH)$_2$D$_3$ and many analogues fail to eliminate the malignant clone [25]. Therefore, the induction of apoptosis should be important for leukemia therapy [26]. The differential control of apoptosis by 1α,25(OH)$_2$D$_3$ in a variety of malignant cells is still not fully understood. Our studies indicated that the 2-methyl and 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues bearing the 1β- with 3α-hy-

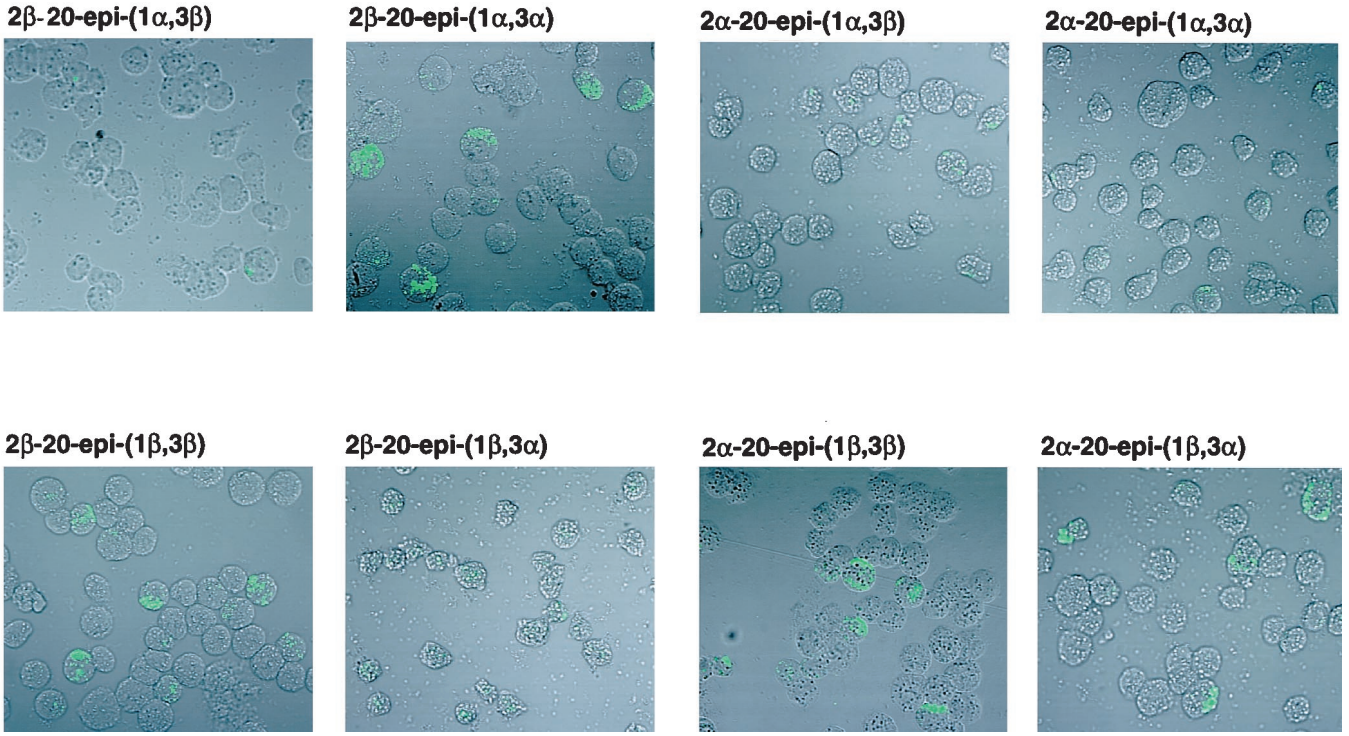


**FIG. 6. Effects of 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues on the appearance of DNA-fragmented cells. The HL-60 cells were treated for 5 days with the 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues ($10^{-8}$ M) and stained with the TUNEL method. The nuclei were visualized by fluorescence microscopy.**

droxyl groups on the A-ring induce apoptosis of HL-60 cells (by nuclear staining, TUNEL staining, DNA gel fragmentation, and caspase-3 activity assays). The caspase-3 activity is known to be negatively regulated by caspase-8 or caspase-9 when cells are induced to undergo apoptosis, whereas the activity is positively regulated when cells are induced to differentiate [27]. We clearly demonstrated that the caspase-3 activities of the analogues capable of inducing

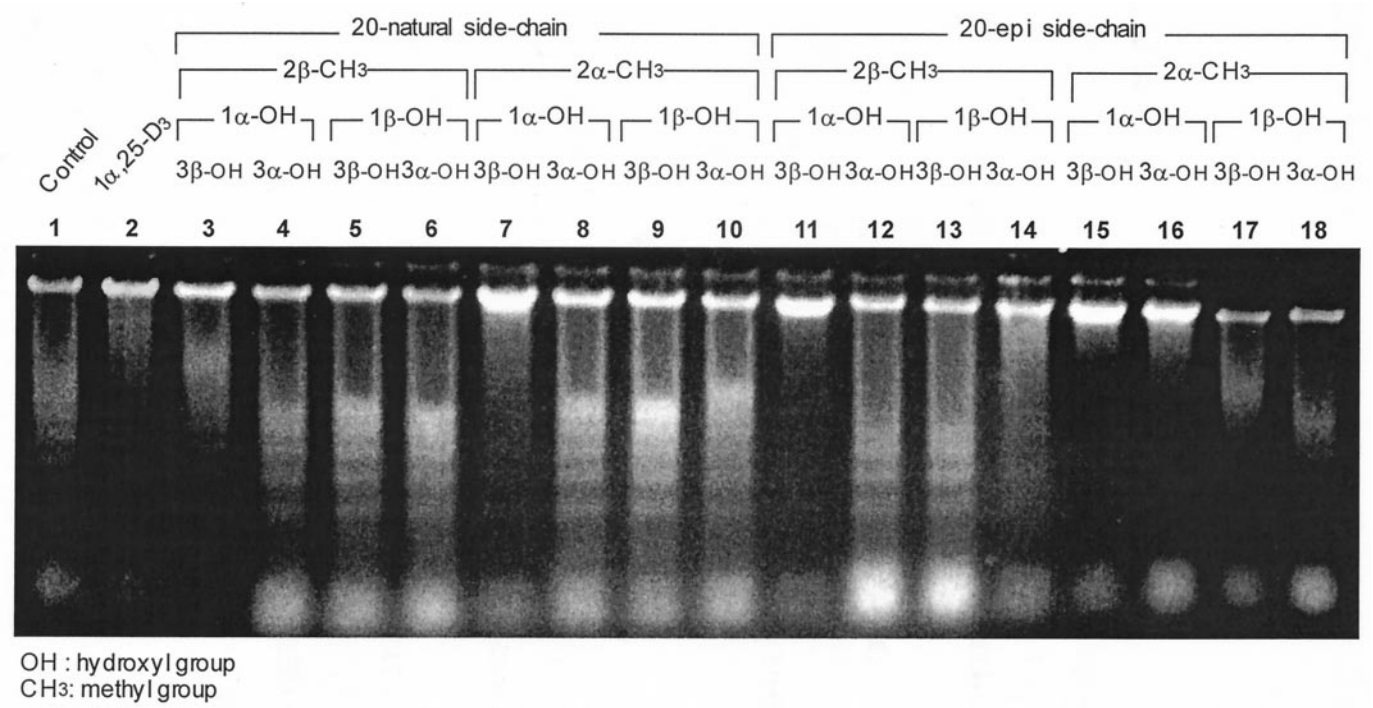


**FIG. 7. DNA fragmentation assay of apoptosis in HL-60 cells treated with 2-methyl or 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues ($10^{-8}$ M) for 5 days. The fragmentation of chromosomal DNA was assessed by electrophoresis on 2.0% agarose gels.**

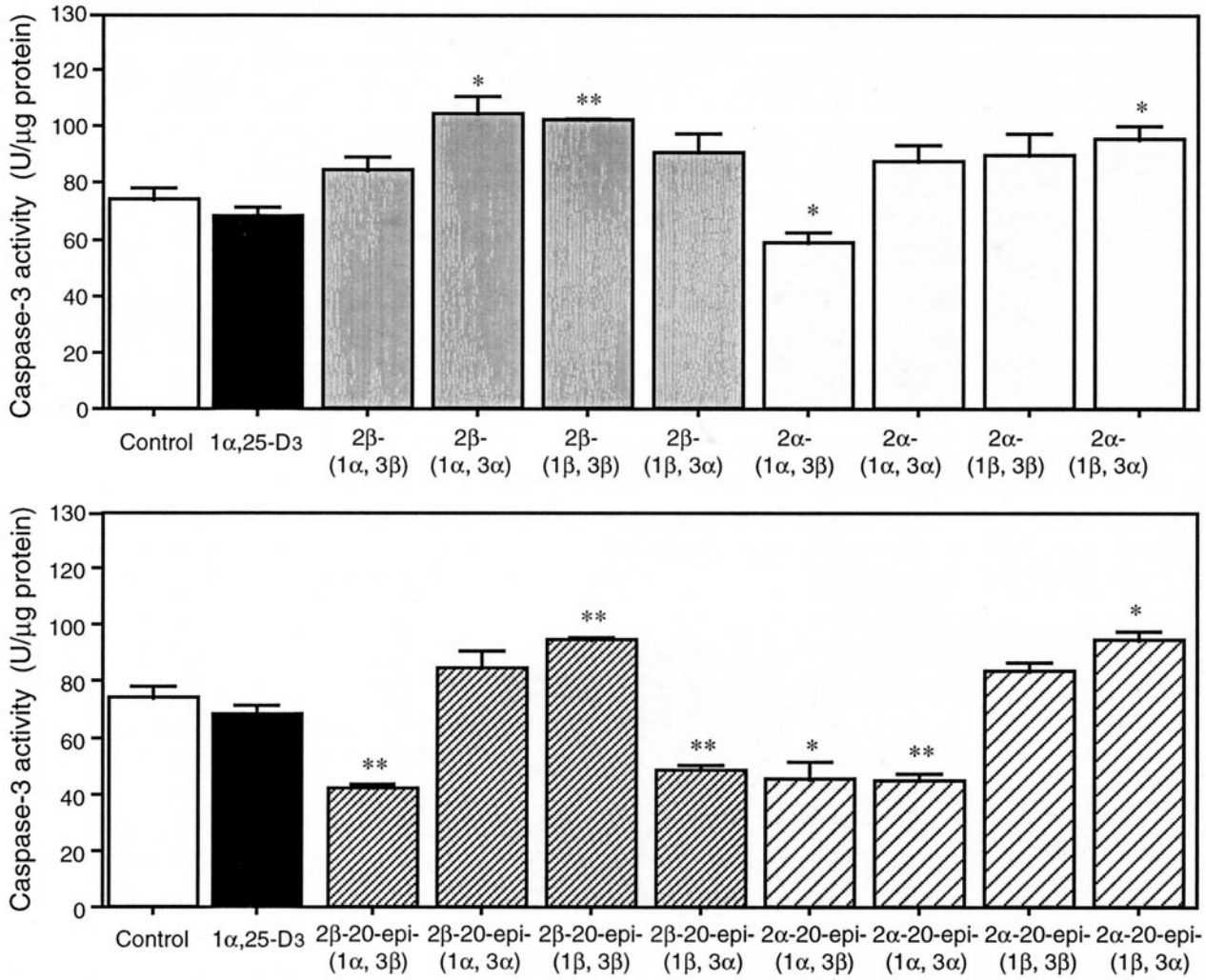


**FIG. 8. Caspase-3 activity in the cell extract from HL-60 cells treated with 2-methyl or 2-methyl-20-epi-1α,25(OH)$_2$D$_3$ analogues ($10^{-8}$ M) for 5 days. Each value represents the mean ± SEM of two independent determinations from three separate experiments. *$P < 0.05$, **$P < 0.01$.**

apoptosis of HL-60 cells were significantly higher, and those of the analogues capable of inducing growth inhibition of HL-60 cells were significantly lower, than those of 1α,25(OH)$_2$D$_3$. These results clearly indicated that there is an inverse relationship between the induction of differentiation and the apoptosis of HL-60 cells by the diastereomers of the 2-methyl-1α,25(OH)$_2$D$_3$ and the 2-methyl-20-epi-1α,25(OH)$_2$D$_3$. It is also noteworthy that the differentiation-inducing potency of the 20-epi analogues 2β-20-epi-(1α,3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3β), and 2α-20-epi-(1α,3α) appears to be greater than that of the 20-natural counterparts 2β-(1α,3β), 2β-(1β,3α), 2α-(1α,3β), and 2α-(1α,3α). It has been reported that 20-epi-1α,25(OH)$_2$D$_3$ is more active than 1α,25(OH)$_2$D$_3$ in stimulating monocytic differentiation of HL-60 cells into macrophages via a VDR-mediated mechanism [28]. The present results suggested that the 20-epi side chain functions as an enhancer of a VDR-mediated signaling pathway and as a suppressor of a VDR-non-mediated mechanism. In addition, it is plausible that the orientations of the C-1 and C-3 hydroxyl groups of the A-ring may be structural motifs responsible for regulating two signaling pathways, namely a VDR-mediated mechanism, such as regulation of cell differentiation and target gene expression, and a non-VDR-mediated mechanism such as apoptosis. This is the first finding of the structure-specific control of differentiation and apoptosis of HL-60 cells by 1α,25(OH)$_2$D$_3$, analogues.

The molecular mechanisms responsible for the growth inhibition, differentiation, and apoptosis of HL-60 cells mediated by the analogues characterized by the orientations of the two hydroxyl groups at positions 1 and 3 of the A-ring are not well understood. It has been reported that 1α,25(OH)$_2$D$_3$ induces apoptosis of breast cancer cell line MCF-7 cells. Initially, the apoptotic effect was induced by increasing the release of apoptosis-inducing factor (AIF) and cytochrome *c* from the mitochondria via suppression of Bcl-2 expression, and subsequently by activating caspase-3 activity, which in turn cleaves the 45-kDa subunit of the DNA fragmentation factor (DFF) to produce an active factor that triggers apoptotic DNA fragmentation [29]. This

apoptotic process is known to be, at least in part, induced via a VDR-mediated signaling pathway. In contrast, it was reported that 1α,25$(OH)_2D_3$ prevents apoptosis of HL-60 cells induced by staurosporin and UV irradiation by increasing Bcl-2 expression. Moreover, it has been shown that the treatment of HL-60 cells with antisense VDR mRNA resulted in apoptosis [30]. From these findings, it is obvious that the antiapoptotic effects of 1α,25$(OH)_2D_3$ on HL-60 cells are also regulated via a VDR-mediated signaling pathway. It is unlikely that the apoptosis-inducing effects of the 2-methyl-1α,25$(OH)_2D_3$ and 2-methyl-20-epi-1α,25$(OH)_2D_3$ analogues are related to a VDR-mediated mechanism because they do not bind VDR at all and do not exhibit VDR-mediated biological activities in HL-60 cells. Norman *et al.* reported that 1β,25$(OH)_2D_3$ exhibited no VDR-mediated biological activities, although it acts as an antagonist on membrane receptor(s) that mediate(s) protein kinase C (PKC) and protein kinase A signaling pathways to modulate calcium channeling and release of calcium from endoplasmic reticulum [31–33]. It has been reported that both *Bcl-2* and *Bcl-XL* gene expressions are regulated via PKC-dependent signaling pathways in many cell types. Since most analogues with apoptosis-inducing activity possess the 1β-hydroxyl group in the A-ring, and in analogy to 1β,25$(OH)_2D_3$, it is plausible that these analogues induce apoptosis of HL-60 cells via binding to membrane VDR. Interaction of the 1α,25$(OH)_2D_3$ analogues with membrane apoptosis mechanisms may be of special physiological significance and provide evidence to understand the membrane receptor-mediated signaling mechanism of 1α,25$(OH)_2D_3$.

In summary, we have found that in the 2-methyl-1α,25$(OH)_2D_3$ and 2-methyl-20-epi-1α,25$(OH)_2D_3$ analogues, the 1β-hydroxyl group and/or the 3α-hydroxyl group of the A-ring appear to be key structures for induction of apoptosis in HL-60 cells. The potent analogues could be clearly divided into two groups, in which those bearing both the 1α- and 3β-hydroxyl groups on the A-ring were potent inducers of differentiation and a G1 arrest of HL-60 cells, while those bearing the 1β-hydroxyl group together with the 3α- or 3β-hydroxyl groups on the A-ring were potent stimulators of apoptosis of HL-60 cells. We have clearly identified for the first time the structural motifs of the stereochemistry at positions 1, 2, 3, and 20 of the 1α,25$(OH)_2D_3$ molecule responsible for the induction of differentiation and apoptosis of HL-60 cells. These findings will provide useful information not only for development of therapeutic agents for the treatment of leukemia and other cancers, but also for structure–function studies of 1α,25$(OH)_2D_3$ analogues.

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