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Vitamin D Analog 25-(OH)-16,23E-Diene-26,27-Hexafluoro-Vitamin D_3 Induces Differentiation of HL60 Cells With Minimal Effects on Cellular Calcium Homeostasis

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Abstract Numerous vitamin D_3 analogs (VDAs) can inhibit the proliferation of cells from several types of human malignancies. The physiologically active form of vitamin D_3, 1,25-dihydroxyvitamin D_3(1,25D_3), is formed by successive hydroxylations of cholecalciferol at the 25 and 1α positions. In this study we examined the effects of the absence of the 1α(OH) group, introduction of a double bond in position 16, and further modifications at the 23, 26, and 27 positions in the side chain on the potency of the VDAs. The parameters studied were the rapidity of the induction of monocytic differentiation, the cell cycle traverse, and the effects of VDAs on intracellular calcium homeostasis in HL60 cells. The results show that (1) 1,25D_3 derivatives which lack the 1α(OH) group have little differentiation-inducing activity, (2) hexafluorination (6F) of the terminal methyl groups in the side chain partially restores the activity of 1α-desoxy compounds and potentiates the activity of 1α hydroxylated compounds, and (3) 25-(OH)-16,23E-diene-26,27-hexafluoro-vitamin D_3 (Ro25-9887) alone among the twelve compounds tested induces differentiation with only minimal changes in the basal levels of intracellular calcium and store-dependent calcium influx in HL60 cells. Addition of 1α(OH) group to this compound increases its differentiation-inducing activity but also elevates basal calcium level. The results suggest that altered calcium homeostasis is not an obligatory component of HL60 leukemia cell differentiation, and that Ro25-9887 and related VDAs may be suitable for testing as components of anti-leukemic therapy. © 1996 Wiley-Liss, Inc.

Key words: vitamin D_3, differentiation, intracellular calcium, store-dependent calcium influx, cell cycle blocks

The principal obstacle to the control of proliferative diseases is the development of life-threatening side effects when compounds which block cell growth and division are used for the therapy of these diseases. Vitamin D analogs (VDAs) are already useful for the treatment of psoriasis [Holick, 1989], a hyperproliferative condition of the skin, but they hold as yet unfulfilled promise for the treatment of neoplastic diseases such as breast, prostate, and colon cancers, as well as myeloid leukemias [Abe et al., 1991; Eisman et al., 1987; Honma et al., 1983; Skowronski et al., 1993]. This promise is based on the observations that growth of these cells in vitro is inhibited by 1,25 dihydroxyvitamin D_3(1,25D_3), the physiologically active form of vitamin D_3 [Holick et al., 1971; Lawson et al., 1971; Myrtle et al., 1970], with evidence of some differentiated characteristics in the treated cells. The induction of the differentiated phenotype by 1,25D_3 and its analogs is especially clearly demonstrable in human promyelocytic leukemia HL60 cells [Abe et al., 1981; Mangelsdorf et al., 1984], which therefore represent a useful model for studies of the differentiation therapy of human malignant diseases.

Since 1,25D_3 is the major regulator of calcium homeostasis in the body [Norman, 1979], it is not surprising that systemic administration of this compound leads to unacceptable levels of hypercalcemia before anti-proliferative concentrations of 1,25D_3 can be achieved [Koeffler et

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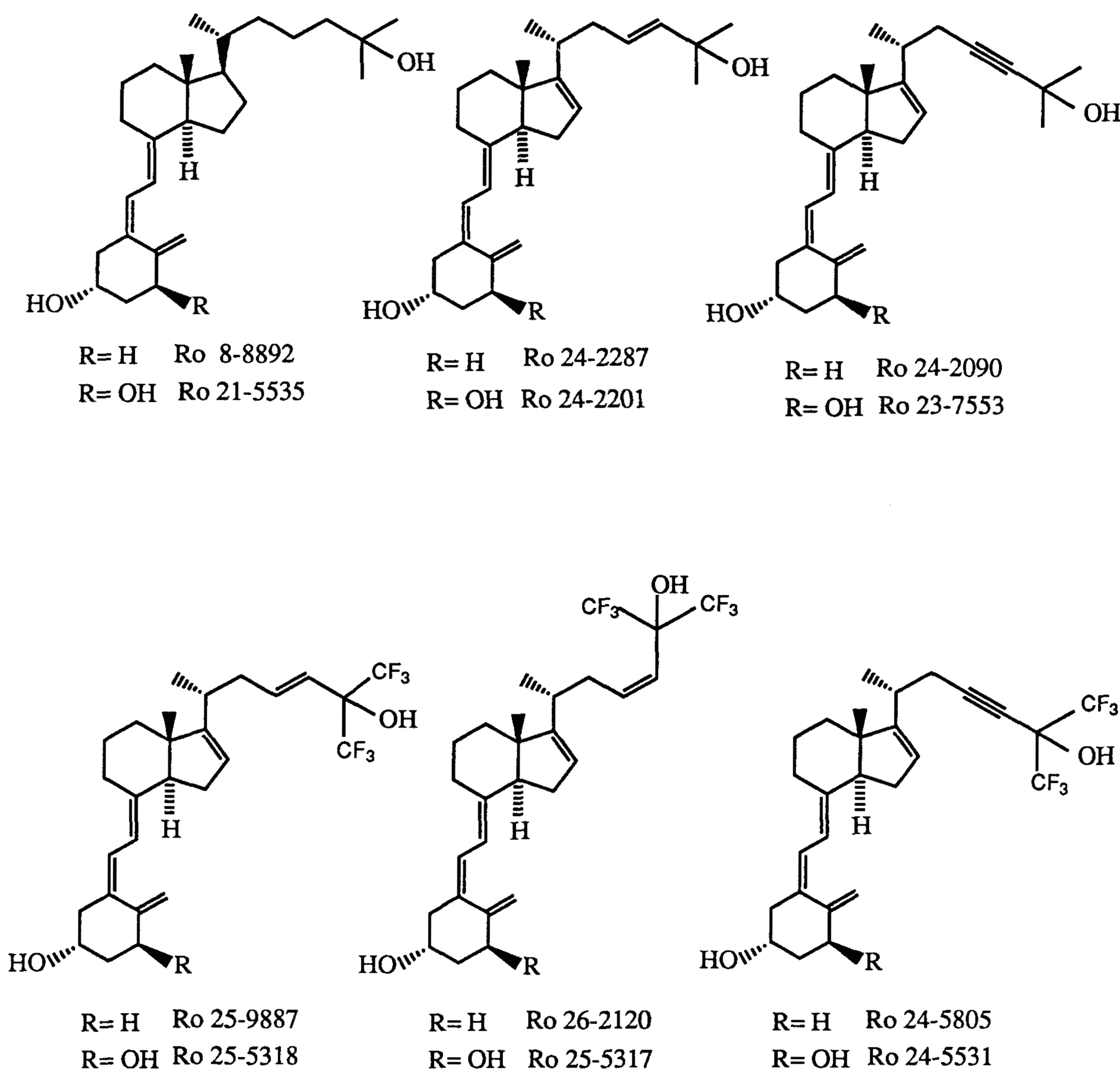

Fig. 1. Structural formulas of $1,25D_3$ (Ro21-5535) and its analogs used in these studies.

al., 1985]. However, modifications of the structure of this secosteroid have resulted in analogs for which some limited success has been reported with regard to the retardation of growth neoplastic cells in animal models [reviewed in Studzinski et al., 1993]. For instance, $1,25(OH)_2$-16-ene-23-yne-cholecalciferol (Ro23-7553) prolonged survival of mice bearing xenografts of myeloid leukemia cells [Zhou et al., 1990], while $1\alpha,24S-(OH)_2$-22-ene-26,27-cyclo-vitamin D_3 and 24a,26a,27a-trihomo-22E,24E-diene-$1\alpha,25(OH)_2$vitamin D_3 [Colston et al., 1992], as well as 22-oxa-$1,25(OH)_2$vitamin D_3 [Oikawa et al., 1991; Abe-Hashimoto et al., 1993], have reduced the growth of breast cancer cells in animal models. More recently, $1\alpha,25-(OH)_2$-16-ene-vitamin D_3 and $1\alpha,25-(OH)_2$-16-ene-23-yne-26,27-hexafluoro-vitamin D_3 have been reported to have anti-leukemic and chemopreventive activities, respectively [Jung et al., 1994; Anzano et al., 1994; Lucia et al., 1995]. Although all these compounds still have significant hypercalcemic activity, they offer the expectation that the right combination of structural modifications of the secosteroid molecule can be found which results in a better dissociation of the calcemic from the differentiation-inducing activity.

A critically important question that so far has not been answered is whether differentiation

can be induced by VDAs in a given cell without altering the Ca^{2+} homeostasis of the differentiating cell. Previous investigations of $1,25D_3$-induced differentiation of HL60 cells showed considerable increases in cytosolic levels of Ca^{2+} ($Ca^{2+}{}_i$) [Hruska et al., 1988], and this was recently confirmed in our laboratories (manuscript in preparation). We also found increased store-dependent calcium influx (SDCI) and depleted calcium stores in $1,25D_3$-treated HL60 cells (manuscript in preparation). If these changes in intracellular calcium regulation are inescapable accompaniments of differentiation, it could indeed be very difficult, if not impossible, to design a non-hypercalcemic VDA with the ability to induce differentiation. In this study, we therefore focused on the effects of the $1\alpha(OH)$ group, the level of unsaturation at the 23 position and the configuration of the double bond in this position, and the replacement of hydrogen atoms with fluorines in the terminal methyl groups of the side chain. While the structure-function implications of these modifications appear to be complex, it seems clear that significantly increased $Ca^{2+}{}_i$ is not necessary for VDA-induced differentiation of HL60 cells.

MATERIALS AND METHODS

Cell Culture

A well-differentiating subline obtained by cloning of ATCC-240 HL60 cells [Studzinski et al., 1986] was grown in suspension culture in RPMI medium supplemented to 1% with glutamine and to 10% with bovine fetal serum. For experiments, the cells were suspended in fresh medium at 3×10^5 cells/ml and fed with fresh medium every 72 h. Antibiotics were not used in the culture medium to avoid the risk of inapparent infection, and mycoplasma contamination was excluded by both autoradiographic [Studzinski et al., 1973] and microbiological monitoring every month.

Vitamin D_3 Analogs

VDAs were synthesized at Hoffmann-LaRoche, Nutley, NJ, according to the general methods described by Baggiolini et al. [1986]. Their formulas are shown in Figure 1.

Demonstration of the Differentiated Phenotype

Mouse anti-human CD11b (Mo1-FITC) and CD14 (MY4-FITC) monoclonal antibodies were obtained from Coulter Corporation (Miami, FL).

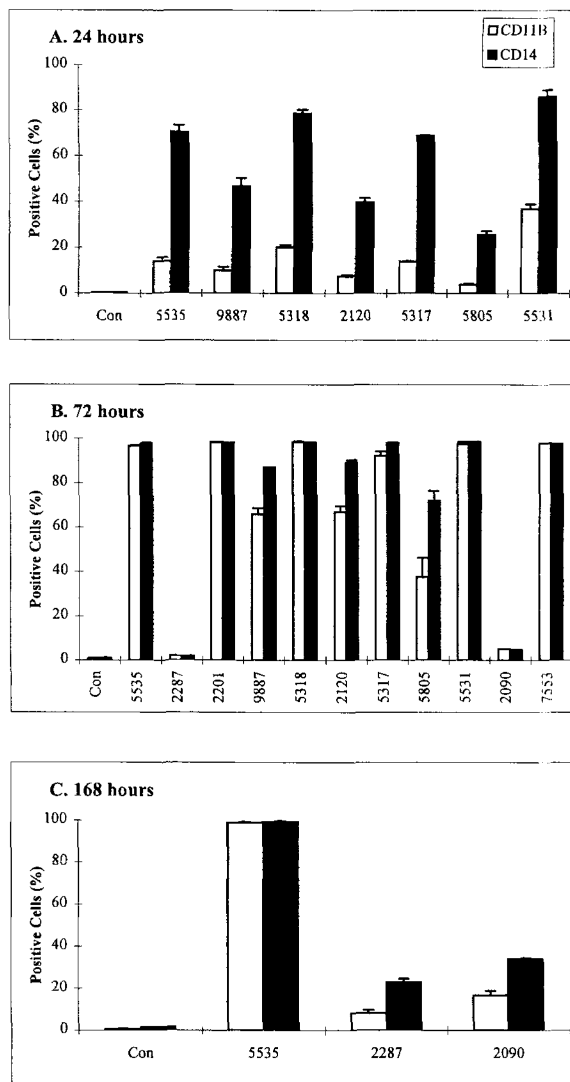

Fig. 2. Expression of surface markers of monocytic differentiation, CD11b (Mo1) and CD14 (My4), by HL60 cells exposed for varying periods of time to ethanol (vehicle control, Con), or to 10^{-7} M concentrations of 11 VDAs. **A:** Expression of the differentiation markers following 24 h exposure to three pairs (1-desoxy and $1\alpha(OH)$ in each pair) of very potent 6F VDAs. Note that the expression of CD14 antigen preceded the expression of CD11b antigen. **B:** Expression of the differentiation markers following 72 h exposure to $1,25D_3$ and to 5 pairs of VDAs. **C:** Expression of differentiation markers following exposure to 168 h to 1-desoxy non-fluorinated compounds. The means ± SE of three experiments are shown. In each panel the effect of $1,25D_3$ (5535) is shown for comparison.

HL60 cells were harvested after treatment with $1,25D_3$ or its analogs, and 1×10^6 cells were spun down at 400*g* for 10 min at 4°C. Cells were washed twice with ice-cold 1 × phosphate-buffered saline (PBS), and 0.5 μl of FITC-labeled antibody was added to each tube and the cells incubated at 4°C for 30 min. Three washes

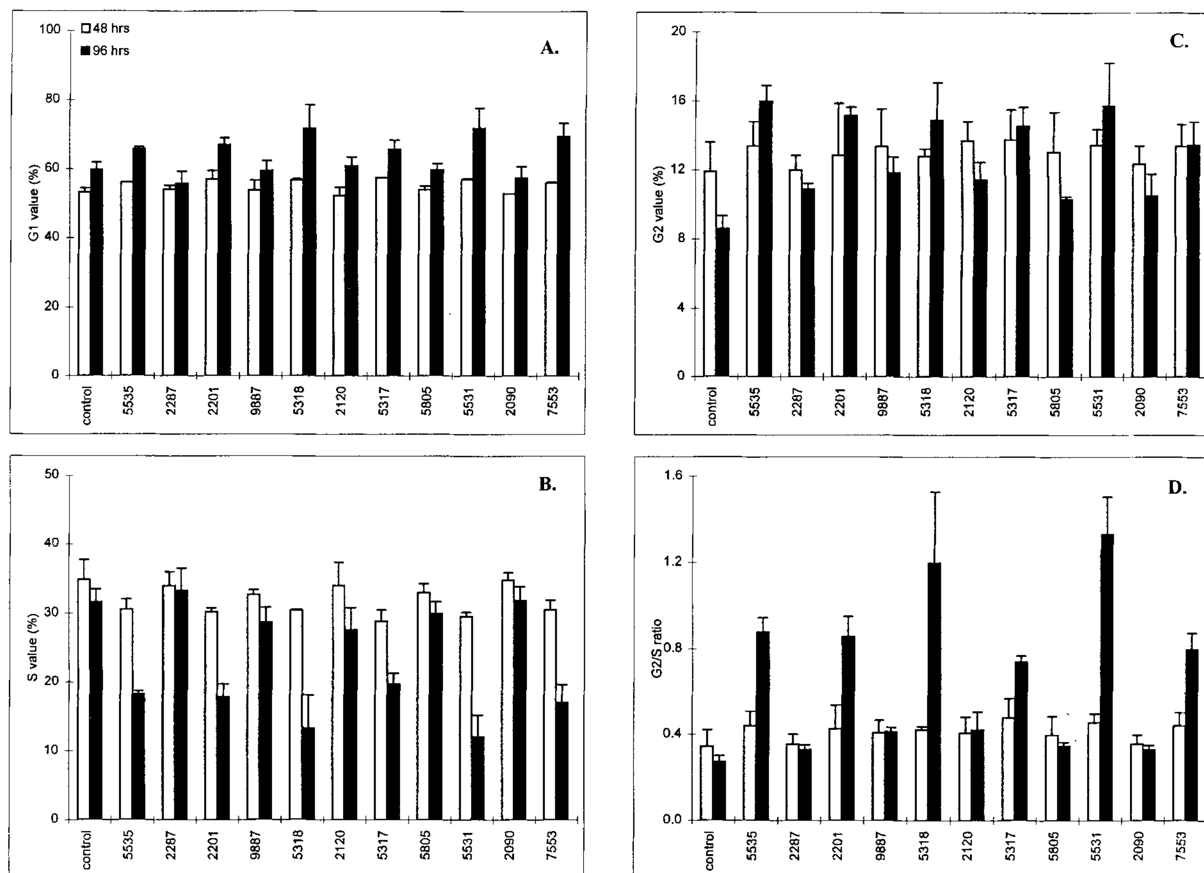

Fig. 3. Flow cytometric determination of the cell cycle distribution of HL60 cells exposed to ethanol, the vehicle control, $1,25D_3$ (5535), and five pairs (1-desoxy vs 1α-OH) VDAs. **A:** Proportions of cells in the G1 phase following 48 or 96 h exposure, as indicated in the chart. **B:** Proportions of cells in the S phase. **C:** Proportions in the G2/M compartment. **D:** Ratio of cells in G2 phase to S phase in each group. The mean values ± SE of three experiments are shown.

with ice-cold 1 × PBS were performed after antibody incubation. The cells were then suspended in 1 ml 1 × PBS and analyzed by flow cytometry on an Epics Profile II instrument (Coulter). Appropriate controls (mouse IgG2b, Sigma, St. Louis, MO) were used in each experiment to set the detection threshold levels.

Cell Cycle Analysis

The detailed procedure has been previously described [Godyn et al., 1994]. Briefly, HL60 cells were harvested at different time points after treatment with $1,25D_3$ or its analogs, and 1 × 10^7 cells were spun down and washed twice with ice-cold 1 × PBS. The cells were fixed in 80% ethanol at −20°C for at least 2 h, then again washed with PBS. Ten units of RNase in 0.5 ml PBS were added, and the cells incubated at 37°C for 30 min. Propidium iodide (PI, 10 μg/ml) in PBS (0.5 ml) was then added to stain the DNA, and the cells were analyzed by flow cytometry using an Epics Profile II instrument (Coulter), and the results were processed using computer Multi-Cycle program (Phenix Flow Systems, San Diego, CA) to obtain the parameters of cell cycle distribution.

Determination of Changes in Calcium Homeostasis

$Ca^{2+}{}_i$ measurements were performed on nonadherent cells as previously described [Balasubramanyam and Gardner, 1995] with minor modifications. Cells were spun down at 180*g* for 10 min, resuspended in HBS (defined below), and incubated at 37°C with 2 μM fura-2 AM (Molecular Probes Inc., Eugene, OR) and 125 μM sulfinpyrazone in N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES)-buffered solution (HBS) consisting of (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and

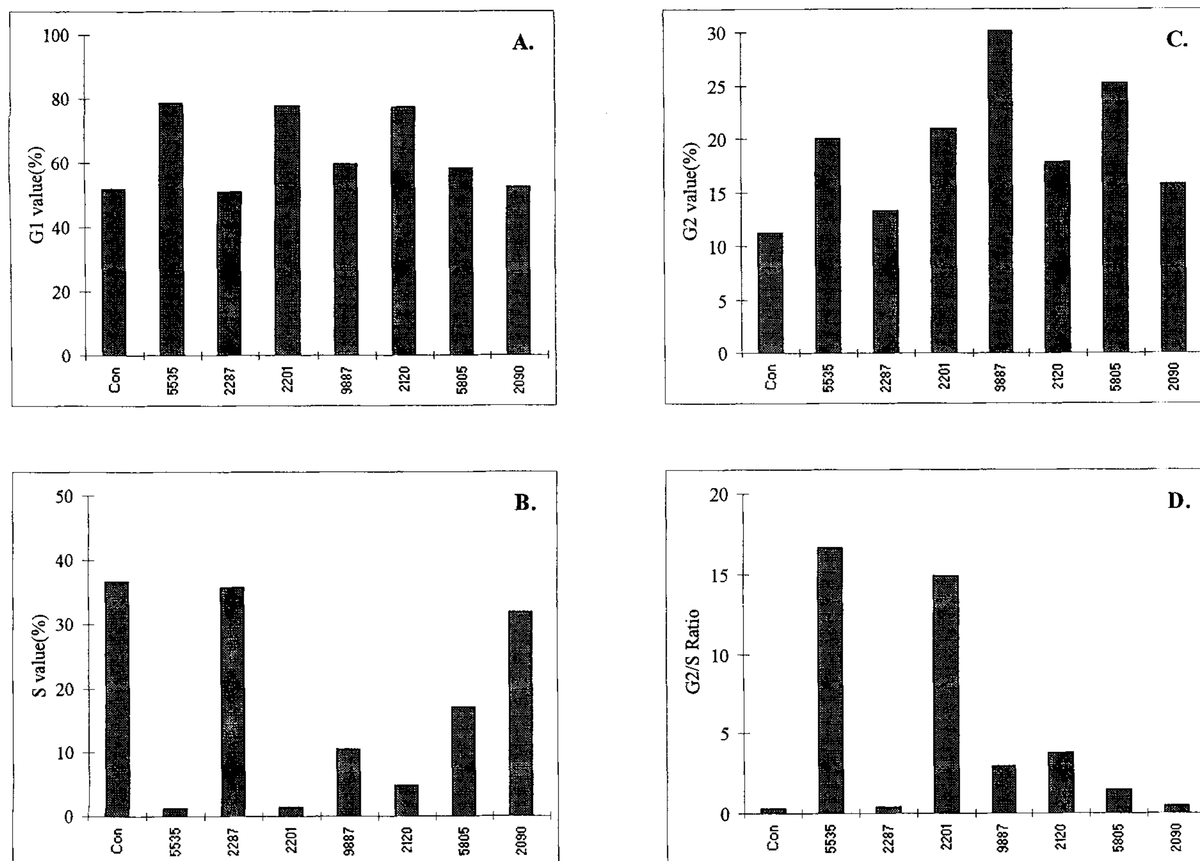

Fig. 4. Flow cytometric determinations of the cell cycle distribution of HL60 cells exposed for 168 h to ethanol control (con), two 1α-OH VDAs (5535 and 2201) and five 1-desoxy VDAs. This experiment was repeated with an essentially identical result. Note that among the 1-desoxy VDAs, the 6F compounds (9887, 2120 and 5805) have produced G1 to S phase and G2 blocks, but non-fluorinated 1-desoxy compounds (2287 and 2090) produced little change in the cell cycle parameters. See Figure 3 legend for definition of A–D.

20 HEPES (pH 7.4) for 30 min at 37°C and distributed (100 μl aliquots of 1.5×10^6 cells) to 500 μl microfuge tubes. Ca^{2+}-free HBS consisted of HBS in which $CaCl_2$ was replaced with 0.3 mM EGTA. Prior to each experiment, cells were centrifuged for 5 s, resuspended in 100 μl of HBS or Ca^{2+}-free HBS, and injected in cuvettes containing 3 ml of the same solution (HBS or Ca^{2+}-free HBS). Fluorescence at excitation wavelength of 340 and 380 nm and emission wavelength at 505 nm was monitored at 37°C with constant stirring in a CM3 spectrofluorometer (SPEX Inc., Edison, NJ). Data were collected at 2 s intervals and $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [1985] as:

$$[Ca^{2+}]_i = K_d(R - R_{min}/R_{max} - R) \times (Sf2/Sb2)$$

where K_d (the dissociation constant for the dye) is assumed to be 224 nM, R is the 340/380 ratio, R_{min} is the ratio in the absence (<1 nM) of Ca^{2+}, R_{max} is the ratio in the presence of saturating Ca^{2+}, and Sf2/Sb2 is the ratio of fluorescence at 380 nm excitation of Ca^{2+}-free fura-2 to Ca^{2+}-bound fura-2. Calibration was achieved by exposing HL60 cells to 100 μM digitonin in the presence of saturating Ca^{2+} (R_{max}) or HBS containing 15 mM EGTA (pH 8.0, R_{min}). Autofluorescence of solutions, drugs, and fura2-free treated cells was subtracted from the 340 and 380 nm fluorescence spectra prior to calculations.

Changes in basal $Ca^{2+}{}_i$ and the peak $Ca^{2+}{}_i$ response of cells in Ca^{2+}-containing medium stimulated with 10 μM N-formyl-methionyl leucyl-phenylalanine (fMLP, Sigma) were monitored for 20 and 100 s, respectively. Thapsigargin (Tg, LC Services, Woburn, MA), a specific inhibitor of the endoplasmic reticulum Ca^{2+} ATPase (SERCA) [Lytton et al., 1991], was used to determine the amount of freely exchangeable

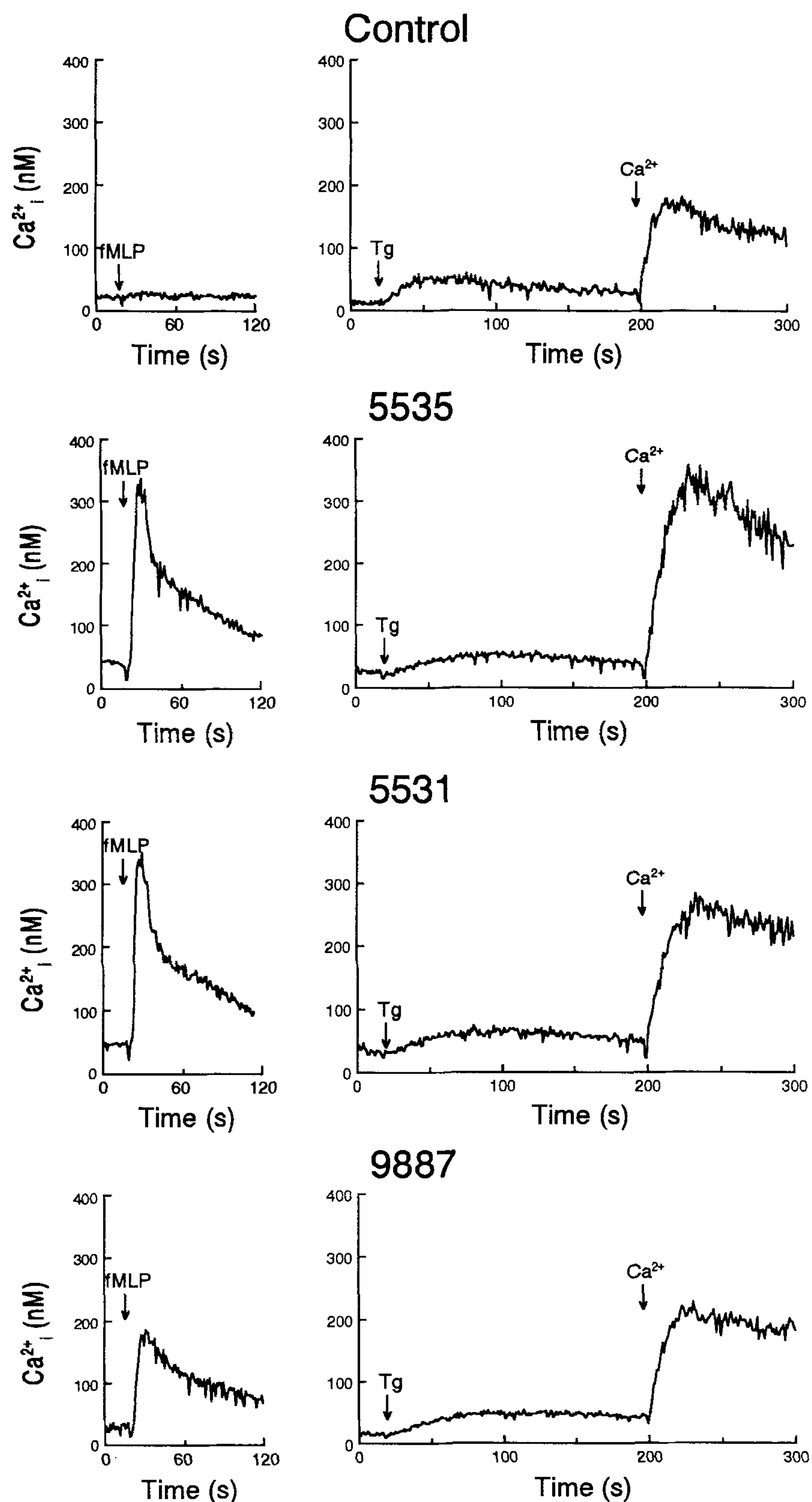

Fig. 5. Representative tracings of the measurements of basal Ca^{2+}_i, fMLP-stimulated change in Ca^{2+}_i, thapsigargin (Tg)-induced change in Ca^{2+}_i, and change in Ca^{2+}_i following addition of Ca^{2+} to medium surrounding the cells with depleted Ca^{2+} stores (SDCI). The left panel in each row represents effects in the presence of extracellular Ca^{2+}, the right panel the effects in the absence of extracellular Ca^{2+}, and the effects of its re-addition at 200 s. The peak increase after re-addition of Ca^{2+} provides the quantitative assessment of SDCI. Pretreatment with VDAs was for 96 h at 10^{-7} M.

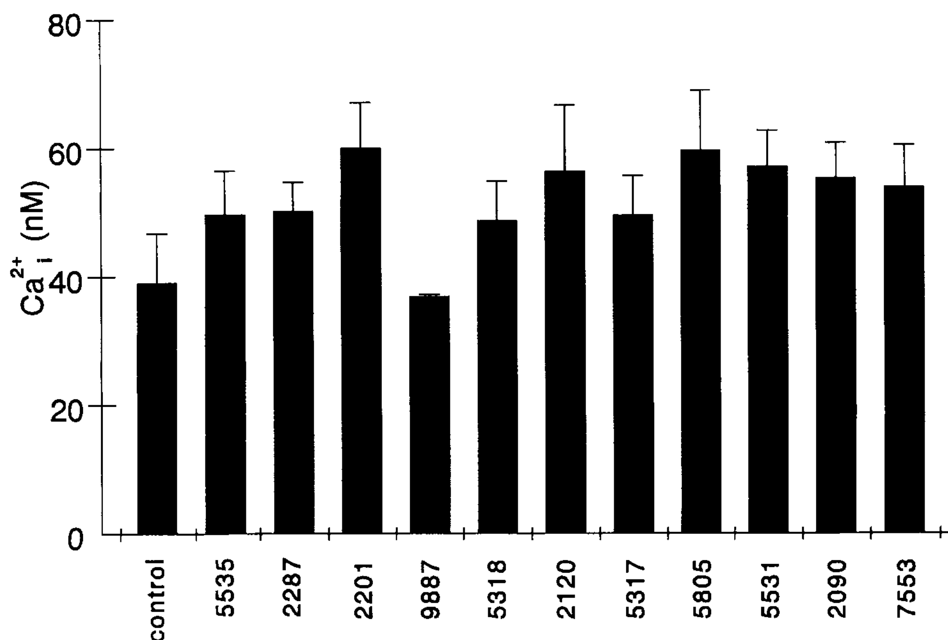

Fig. 6. Basal Ca^{2+}_i levels in HL60 cells exposed for 96 h to the indicated VDAs at 10^{-7} M. Mean values ± SE of three experiments are shown.

Ca^{2+} in intracellular Ca^{2+} stores. This parameter was determined as the peak difference between the Tg-elicited Ca^{2+}_i response and basal Ca^{2+}_i (obtained in Ca^{2+}-free HBS). Depletion of the intracellular Ca^{2+} stores by Tg activates a well-described Ca^{2+} influx pathway known as capacitative Ca^{2+} entry [Putney, 1991] or SDCI [Clementi et al., 1992].

We determined the capacity for SDCI in HL60 cells by two separate methods: (1) the peak increase in Ca^{2+}_i following extracellular Ca^{2+} readdition to cells treated with Tg for 180 s in Ca^{2+}-free HBS; and (2) the rate of Mn^{2+} entry following Tg or fMLP stimulation. Mn^{2+} can serve as a Ca^{2+} surrogate to study Ca^{2+} entry in fura-2 loaded cells [Hallam and Rink, 1985]. In these experiments, Mn^{2+} entering the cell binds to fura-2 and quenches its fluorescence; the quenching is monitored at the isobestic wavelength (360 nm) of fura-2 such that changes in cytosolic Ca^{2+} do not affect the emission fluorescence. Mn^{2+} uptake measurements were performed in fura-2 loaded cells in Ca^{2+}-HBS containing 0.3 mM $MnCl_2$, added 10 s prior to the agonist. Fluorescence was monitored at excitation and emission wavelengths of 360 and 505 nm, respectively, and the results were normalized using 360 nm values obtained immediately before the addition of Tg (100 nM) or fMLP (10 μM). Comparisons of the enhanced decline in fluorescence over control (Mn^{+} influx rates in the absence of Tg or fMLP) were made at 80 and 180 s after agonist addition.

RESULTS

Induction of Monocytic Phenotype by VDAs

HL60 cells exposed to $1,25D_3$ or its analogs differentiate towards the monocyte/macrophage phenotype [Abe et al., 1981; Zhang et al., 1994], and this phenotype can be detected by criteria that include morphological changes, development of non-specific esterase activity, the ability to phagocytose particles from the external milieux [e.g., Studzinski et al., 1985], and the appearance of surface antigens characteristic of differentiated cells in the myeloid-monocytic lineages. In this study we have used two commonly used surface markers; CD14, a monocyte-specific marker [Wright et al., 1991], and CD11b,

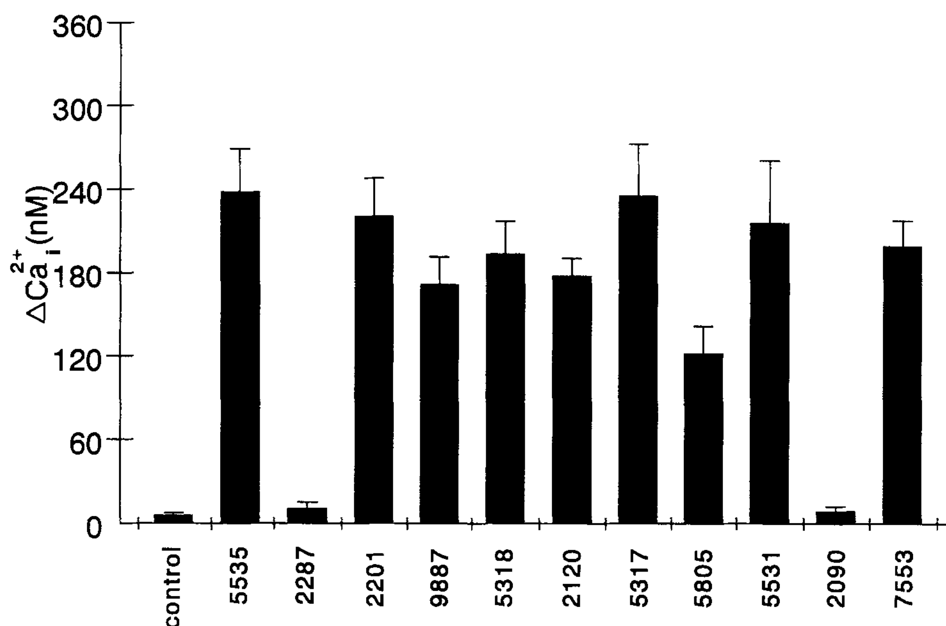

Fig. 7. FMLP-stimulated changes in Ca^{2+}_i following exposure for 96 h to the indicated VDAs at 10^{-7} M. The mean ± SE of three experiments are shown.

characteristic of both monocytic and myeloid lineages [Griffin et al., 1981]. Also, since the effects of VDAs are both concentration- and time-dependent, we fixed the concentration of VDAs at 10^{-7} M, which is near-maximal for $1,25D_3$ [Zhang et al., 1994], and followed the kinetics of the appearance of these differentiation markers.

Preliminary experiments established that VDAs lacking the 1α OH group and not hexafluorinated at the 26,27 positions have very low differentiation-inducing activity, with $25(OH)D_3$ being inert in the system studied here (data not shown). When differentiation was assessed after 24 h exposure to VDAs with 6F substitutions, however, these were noted to have considerable activity in the absence of the 1α(OH) group (Fig. 2). These data also show that when 1α (OH) group is present and the VDA is hexafluorinated, the 23-yne modification (Ro24-5531) increases its potency in comparison to the 23-double bond compounds, but paradoxically, when the 1α(OH) is absent and 6F present (Ro24-5805), the 23-yne group reduces the potency (Fig. 2A and B). The configuration of the double bond did not appear to make a significant difference to differentiation-inducing potency (Ro25-9887 and Ro26-2120), but hexafluorinated VDAs were more potent than the corresponding compounds without 6F (Ro25-9887 vs. Ro24-2287, and Ro24-5805 vs. Ro24-2090, Fig. 2B).

By 72 h of exposure to VDAs all six 1α(OH) compounds used in this study (Ro24-5535, Ro24-2201, Ro25-5318, Ro25-5317, Ro24-5531, and Ro23-7553) have induced the differentiated phenotype, shown by the expression of both CD11b and CD14 antigens, in all cells in culture. Cultures treated with the corresponding 1α-desoxy 6F VDAs (Ro25-9887, Ro26-2120, and Ro24-5805) contained 70–90% of cells positive for CD14 (Fig. 2B), and at 96 h approximately 95% of these cells were CD14 positive (data not shown), indicating that these compounds are also potent differentiating agents. In contrast, VDAs lacking both the 1α(OH) and 6F groups (Ro24-2287 and Ro24-2090) induced only a small proportion of cells to differentiate, even after 7 days of exposure (Fig. 2C).

Effects of VDAs on the Cell Cycle Traverse

Induction of differentiation markers by treatment of HL60 cells with VDAs occurs in the

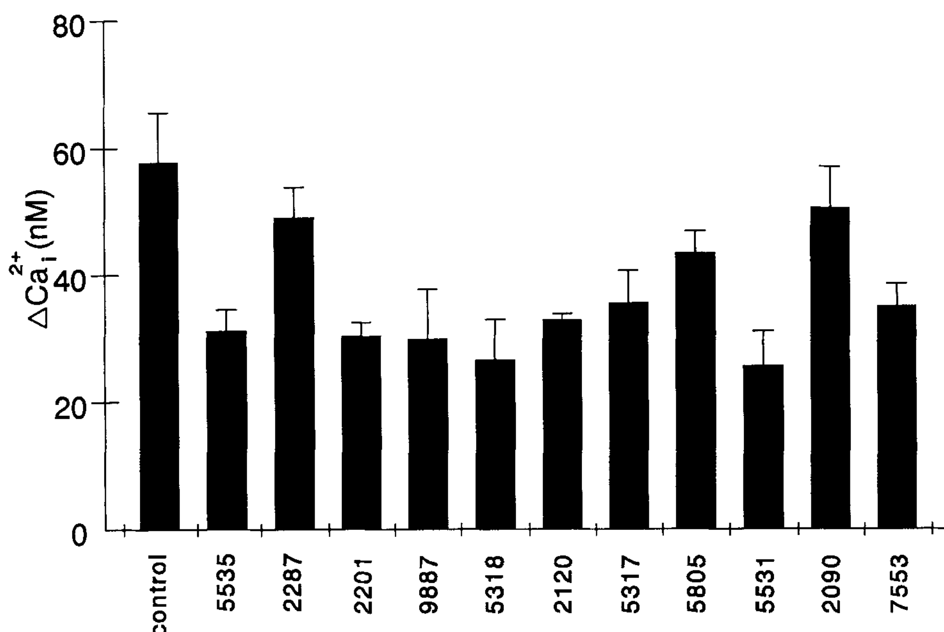

Fig. 8. Thapsigargin-induced changes in $Ca^{2+}{}_i$ in HL60 cells following exposure for 96 h to the indicated VDAs at 10^{-7} M. The mean ± SE of three experiments are shown.

presence of actively proliferating cells [Zhang et al., 1994], but the differentiated cells later exit the cell cycle due to p27/KIP1-induced G1/S phase block [Wang et al., 1996], and a less well-characterized G2 block [Godyn et al., 1994]. We have examined the cell cycle distribution of HL60 cells exposed to $1{,}25D_3$ and to five pairs of VDAs for 48, 96, and 168 h. Figure 3 shows that after 48 h of VDA exposure there was no significant change in the proportion of cells in the G1, S, and G2/M phases of the cell cycle, even though already at 24 h the majority of cells displayed the surface markers of differentiation (Fig. 2A). However, when examined at 96 h all compounds except those lacking both 1α(OH) and 6F groups showed G1 to S phase block in a rough proportion to their differentiation-inducing potency (Fig. 3). In addition, the previously reported G2 block, best evident as the ratio of cells in G2 relative to the cells in S phase (from which the cells enter the G2 phase), was increased by all 1α(OH)-containing compounds. This was especially marked for 6F-containing compounds Ro25-5318 and Ro24-5531 (Fig. 3).

Prolongation of the treatment of HL60 cells with VDAs to 168 h resulted in more complete G1 to S phase and G2 phase blocks when active VDAs were employed, but compounds Ro24-2287 and Ro24-2090 still showed little or no effect (Fig. 4). Thus, in this series of analogs significant anti-proliferative activity requires the presence of either the 1α(OH) or the 6F group on the VDA.

Calcium Homeostasis in VDA-Treated HL60 Cells

In parallel with the determinations of the differentiation-inducing potencies of the VDAs on HL60 cells, we measured several parameters of intracellular calcium homeostasis in cells treated with each compound for 96 h. Figure 5 shows representative tracings obtained in one of the three experiments performed for this purpose. As summarized in Figure 6, basal $Ca^{2+}{}_i$ became elevated by the exposure to the VDAs up to 50% above its ambient level. Interestingly, compound Ro25-9887 produced no detectable increase in $Ca^{2+}{}_i$, even though compounds with little differentiation-inducing activity (Ro24-2287 and Ro24-2090) did increase $Ca^{2+}{}_i$ (Fig. 6). The extent of $Ca^{2+}{}_i$ response to fMLP, an agonist of phagocytic activity, was consistent with

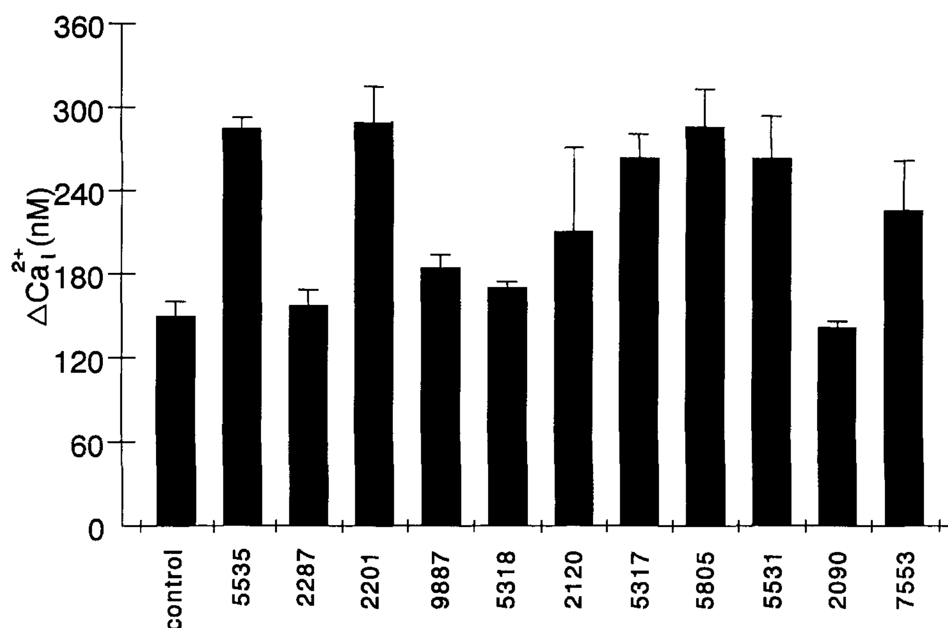

Fig. 9. Store-dependent calcium influx (SDCI) in HL60 cells following exposure for 96 h to the indicated VDAs at 10^{-7} M. The mean ± SE of three experiments are shown.

the differentiation level of the treated cells (compare Figs. 2 and 7), though the VDAs with increased differentiation-inducing potency produced no greater $Ca^{2+}{}_i$ transients than the parent compound $1,25D_3$ (Ro24-5535). Calcium store sizes were measured by the magnitude of the Tg-induced change in $Ca^{2+}{}_i$, and as recently observed (Gardner et al., manuscript in preparation), the intracellular $Ca^{2+}{}_i$ store size of HL60 cells treated with $1,25D_3$ was markedly decreased (Fig. 8). Similar decreases were noted following treatment with the differentiation-inducing VDAs (Fig. 8). In contrast, there were marked discrepancies between VDA's differentiation-inducing potencies and the SDCI measured when extracellular Ca^{2+} was added to cells with depleted calcium stores; i.e., treatment with $1,25D_3$, or the potent differentiation-inducing analogs, resulted in markedly increased SDCI, but treatment with Ro25-9887 and Ro25-5318 did not (Fig. 9). Since both of these latter compounds induce differentiation, especially Ro25-5318, this suggests that this form of Ca^{2+} influx is not essential for monocytic differentiation.

In order to confirm that calcium influx is not significantly increased following exposure to the two "16,23E-diene" compounds (Ro25-9887 and 5318), we measured this parameter using an alternative procedure. This method utilizes manganese (Mn^{2+}) as a surrogate for Ca^{2+}, and since Mn^{2+} is not exchangeable with extracellular medium, this protocol gives results which are somewhat easier to interpret. Using fMLP or Tg, the "16,23E-diene" compounds induced minimal Mn^{2+} entry (Fig. 10A and B), consistent with the SDCI result (Fig. 9). It was also apparent that structural modifications which enhance differentiation-inducing potency of the VDAs do not increase Ca^{2+} influx over the influx resulting from pretreatment with $1,25D_3$. Thus, differentiation and the calcium homeostatic effects of VDAs can be dissociated in this system.

DISCUSSION

Examination of twelve analogs of vitamin D_3 for differentiation and intracellular calcium enhancing potencies has identified 25-(OH)-16,23E-diene-26,27-hexafluoro-cholecalciferol (Ro25-9887) as a strong candidate for in vivo studies of its anti-leukemic efficacy. In our studies, Ro25-9887 induced monocytic differentiation in practically all HL60 cells over a 96 h

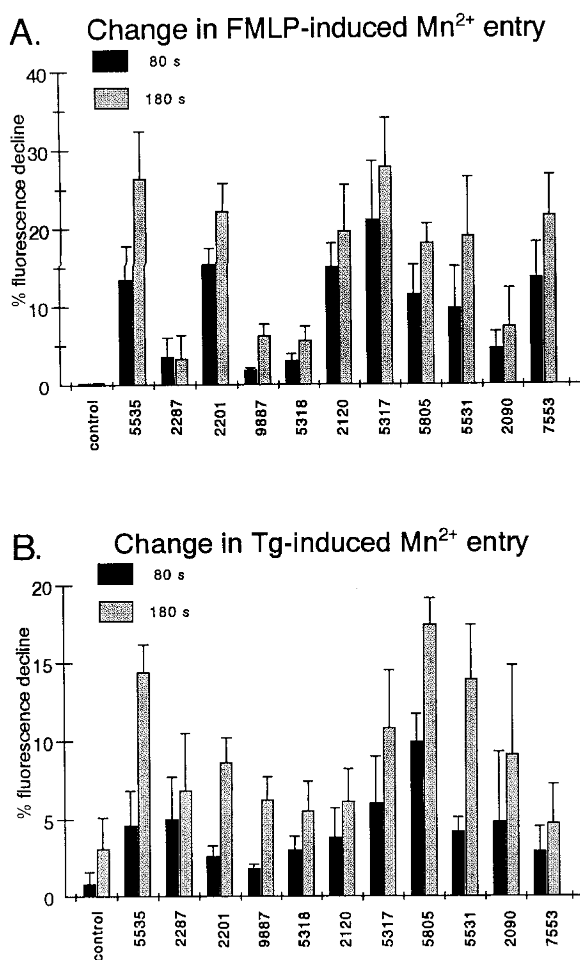

Fig. 10. Changes in Mn entry into HL60 cells following exposure for 96 h to the indicated VDAs at 10^{-7} M. **A:** Mn entry induced by FMLP. **B:** Mn entry induced by Tg. The mean ± SE of three experiments are shown.

period, with no detectable increase in basal $Ca^{2+}{}_i$ levels. FMLP-induced $Ca^{2+}{}_i$ transients were evident, indicative of the differentiated state, and Ca^{2+} stores were depleted, apparently a part of the VDA-induced monocytic phenotype, but three different types of measurements showed that $Ca^{2+}{}_i$ influx was not increased. This property was shared with its 1α-hydroxylated derivative, Ro25-5318, which however did increase basal $Ca^{2+}{}_i$ level, and was a much more powerful differentiating agent.

The demonstration of the absence of a significant elevation of $Ca^{2+}{}_i$ in HL60 cells undergoing Ro25-9887-induced differentiation is important since it shows that it is possible to dissociate these two events. However, it does not exclude that Ro25-9887 may target other cell types to produce hypercalcemic effects. Thus, studies of additional structural features in the secosteroid molecule should prove to be important for cell specificity of its actions.

In spite of its high differentiation potency, Ro25-5318 may be a less attractive candidate than Ro25-9887 for an antineoplastic therapeutic agent, because of its tendency to induce relatively more alterations in intracellular calcium homeostasis in HL60 cells, and to increase the G2/S ratio (Fig. 3D). The concern is that this ratio, also highly elevated by Ro24-5531, may be indicative of G2 phase abnormalities, which occasionally lead to mitotic errors and result in a higher ploidy cell population [Zhang et al., 1996; Wajchman et al., 1996]. This, of course, usually results in the progression to more aggressive malignancy in human neoplastic diseases.

This survey of six pairs of VDAs also showed that the 1α(OH) group was not essential for differentiation-inducting activity if the hydrogens in the terminal methyl groups of the side chain were replaced by fluorines. In vitro tests of VDA binding to vitamin D receptor (VDR) show low binding affinity for ligands not containing the 1α(OH) group [reviewed by Bouillon et al., 1995]; however, the VDR bound to its cognate DNA element (VDRE) appears to have altered binding affinities [Cheskis et al., 1995], so it is possible that side chain modifications of the ligands may result in VDAs which enhance VDR's transactivation/transrepression functions, even in the absence of the 1α(OH) group.

The concept of several forms of activated VDR activation by ligands with different structural modifications can also be invoked to explain why the "16,23E-diene" VDAs induce relatively low Ca^{2+} influx and little increase in the basal $Ca^{2+}{}_i$ level. If ligands differentially alter the three-dimensional structure of the VDR protein, the alternative forms of activated VDR may have different combinatorial properties for its transcription-regulating partners such as RXR, or co-activator/co-repressor proteins, which interact with the basic transcriptional machinery. It is thus possible that a yet to be identified gene which regulates the SDCI in HL60 cells is not activated by the "16,23E-diene" ligand-bound VDR, but is activated by VDR bound by the other potent VDAs. Elucidation of these mechanisms will be crucial to further advances in the development of effective anti-leukemic therapy that includes derivatives of vitamin D.

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