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Relationship between Differentiation-inducing Activity and Hypercalcemic Activity of Hexafluorotrihydroxyvitamin D_3 Derivatives

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Abstract. *Among 16 newly synthesized hexafluorotrihydroxyvitamin D_3 derivatives, 24-Homo-26,26,26,27,27,27-hexafluoro (24H-F_6)-1,24(S), 25(OH)$_3$ vitamin D_3 (VD_3)(DD-011)**[16]** induced differentiation (i.e., appearance of NBT-positive cells) of human promyelocytic leukemic HL-60 cells most efficiently (EC_{50}=0.5 nM), followed by 24H-F_6-1,25(OH)$_2$-22-oxa-VD_3 (DD-006) **[11]** > F_6-1,25(OH)$_2$-VD_3 (F6VD3) **[2]** > F_6-1,25(OH)$_2$-22-ene-VD_3 (DD-009) **[14]** > 24H-F_6-1,25(OH)$_2$-VD_3 (F6C28) **[3]** > 24H-F_6-1,25(OH)$_2$-1,23(S),25(OH)$_3$-VD_3 (DD-015) **[18]** > 24H-F_6-1,25(OH)$_2$-22-ene-VD_3 (mvd1400) **[6]** > 22H-F_6-1,25(OH)$_2$-24-ene-VD_3 (mvd3400) **[5]** > 24H-F_6-1,23(R),25(OH)$_3$-VD_3 (DD-014) **[17]** > 24H-F_6-1,22(S),25(OH)$_3$-VD_3 (DD-003) **[7]** > 24H-F_6-1,22(S),25(OH)$_3$-24-yne-VD_3 (DD-005) **[9]** > 24H-F_6-1,22(R),25(OH)$_3$-24-yne-VD_3 (mvd-1235) **[10]** > F_6-1,25(OH)$_2$-22-ene-VD_3 (DD-008) **[13]** = 1,25(OH)$_2$$VD_3$ **[1]** (CC_{50}=6 nM). On the other hand, 24H-F_6-1,22(R),25(OH)$_3$-VD_3 (DD-004) **[8]**, which is an isomer of DD-003 **[7]**, showed much reduced activity (CC_{50}=100 nM), suggesting the importance of the configuration of the OH group at the C-22. When their differentiation-inducing activity was plotted vs. the octanol-water partition coefficient (log P) used as a parameter of hydrophobicity, a bell-shaped curve was produced, with the bottom at log P=5.4-5.8. There was no clear-cut relationship between the differentiation-inducing activity and hypercalcemic activity (serum calcium elevating activity). Compounds **[3, 7, 11, 17]** showed relatively higher differentiation-inducing activity, with lesser hypercalcemic activity, as compared with **[1]**. Administration of **[7]** showed potent antiproliferation activity against colon cancer transplanted in nude mice. These results further confirmed the antitumor potential of hexafluorotrihydroxyvitamin D_3 derivatives.*

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Key Words: Hexafluorotrihydroxyvitamin D_3 derivatives, differentiation, hypercalcemic activity, structure-activity relationship, log P, hydrophobicity.

Since the discovery of the differentiation-inducing activity of the activated form of vitamin D_3, 1α,25-dihydroxycholecalciferol (1,25(OH)$_2$$VD_3$) against mouse myeloid leukemia cells M1 into maturing macrophages (1), a variety of newly synthesized vitamin D_3 derivatives have been tested for their antitumor potential, in addition to their therapeutic activity against bone diseases such as osteoporosis.

1,25(OH)$_2$-16-*ene*-5,6-*trans*-VD_3, which has a double bond at the C-16 position of 1,25(OH)$_2$$VD_3$, showed 10-100 times higher antiproliferative activity against prostate cancer (LNCaP), breast tumor (MCF-7) and promyelocytic leukemia (HL-60) cell lines and lower hypercalcemic activity (serum calcium elevating activity) than 1,25(OH)$_2$$VD_3$. This compound enhanced the expression of cyclin-dependent kinase inhibitors (CDKIs), such as $p21^{waf1}$ and $p27^{kip1}$, inhibited the expression of telomerase, and induced the increase in G0-G1-phase cells and the decrease in S-phase cells (2).

(23*S*)-25-dehydro-1α-hydroxyVD_3-26,23-lactone and (23*R*)-25-dehydro-1α-hydroxyVD_3-26,23-lactone, which has a lactone ring at C-23, 26 of 1,25(OH)$_2$$VD_3$, bound to the vitamin D_3 receptor (VDR) more tightly than did the corresponding natural form, (23*S*,25*R*)-1α,25(OH)$_2$$VD_3$-26,23-lactone, but rather inhibited the differentiation-inducing activity of 1,25(OH)$_2$$VD_3$ (3). 24-Oxo metabolites of vitamin D_3 analogues showed significantly lower hypercalcemic activity, maintaining their differentiation-inducing activity (4).

The hexafluorinated vitamin D_3 analogs, 26,26,26,27,27,27-hexafluoro-1,25(OH)$_2$$VD_3$ showed 10 times higher differentiation-inducing activity against HL-60 cells than 1,25(OH)$_2$$VD_3$, possibly due to weaker

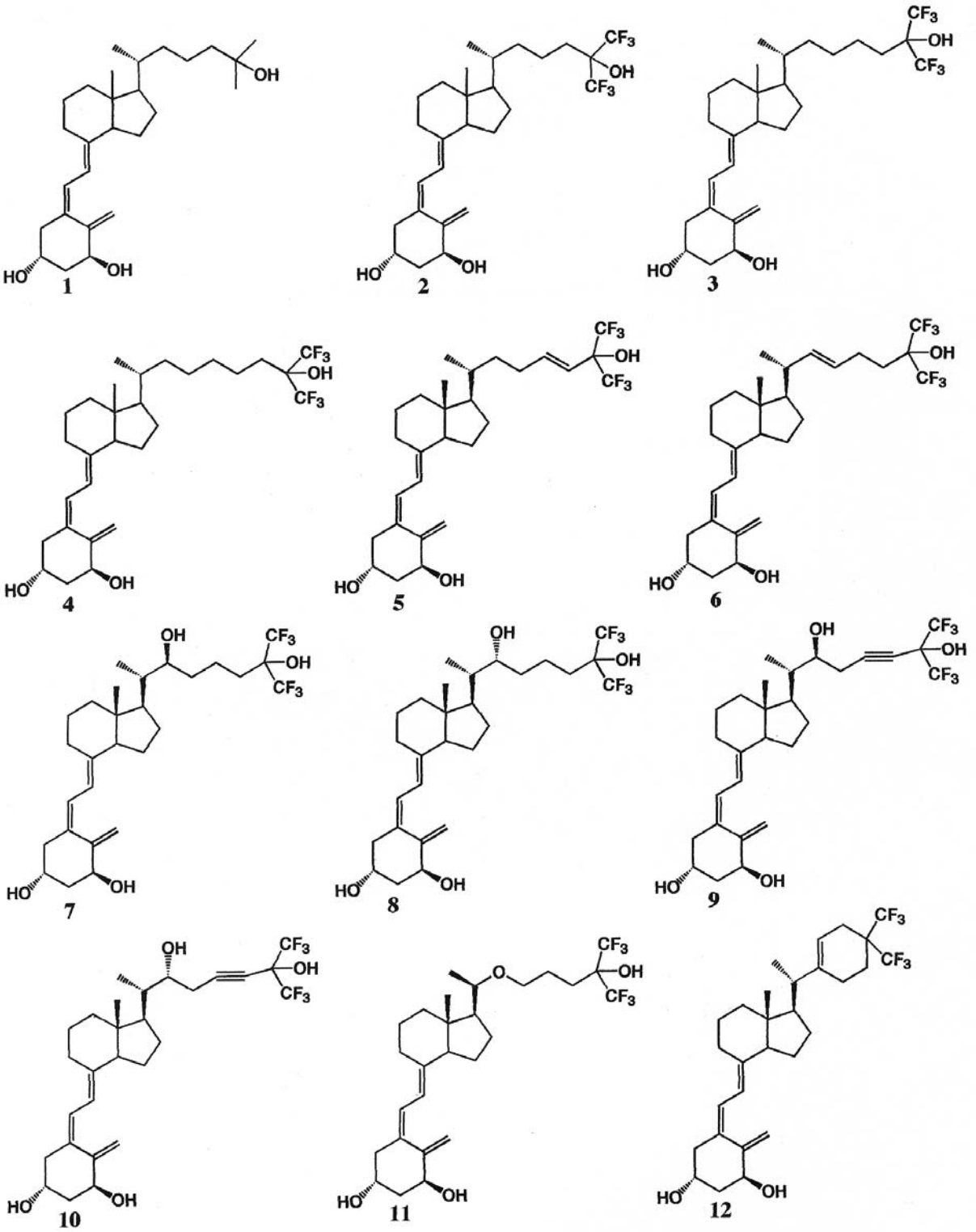

Figure 1

Figure 1 *continued*

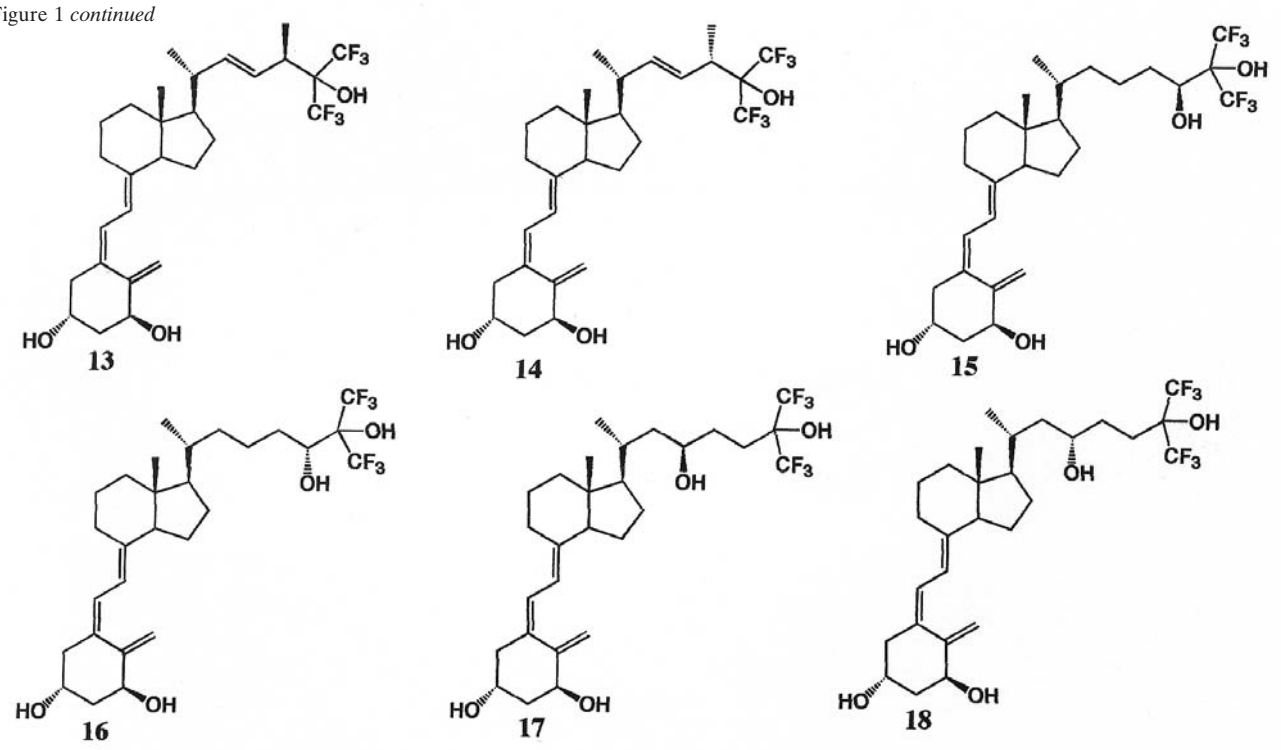

Figure 1. *Structure of hexafluorotrihydroxyvitamin D_3 derivatives.*

binding to serum vitamin D binding protein and decreased metabolic inactivation (5). Although this compound induced the differentiation of human leukemic cells and colonic carcinoma cells HT-29 in combination with NaB, it showed strong hypercalcemic activity, making its application as an anticancer drug difficult (6). The introduction of the cyclopropyl group at C-20 of fluorinated vitamin D_3 significantly enhanced the antiproliferative/differentiation-inducing activity against prostate cancer, breast cancer and HL-60 leukemic blast cells, and increased the ratio of G1-phase cells/S-phase cells. Administration of this compound (0.005-0.01 μg/mouse) resulted in significant tumor regression, with minor hypercalcemic effect (7).

Although the fluorination of VD_3 derivatives has generally elevated the differentiation-inducing activity, the relationship between the differentiation-inducing activity (good effect) and the hypercalcemic activity (adverse effect) remains unclear. We investigated here both the differentiation-inducing activity against HL-60 cells and the hypercalcemic activity of 16 newly synthesized vitamin D_3 derivatives derived mostly from compound **[3]**, which has a longer side chain than compound **[2]** by the introduction of one more carbon (Figure 1).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RPMI1640 medium (GIBCO BRL, NY, USA); fetal bovine serum (FBS), nitroblue tetrazolium (NBT), tetradecanoylphorbol 13-acetate (TPA) (Sigma Chem. Co., St. Louis, MO, USA). A total of 18 vitamin D_3 derivatives: 1,25$(OH)_2VD_3$ **[1]**, 26,26,26,27,27,27-hexafluoro (F_6)-1,25$(OH)_2$-VD_3 (F6VD3) **[2]**, 24-*Homo* (24H)-F_6-1,25$(OH)_2$-VD_3 (F6C28) **[3]**, 23,24-diH-F_6-1,25$(OH)_2$-VD_3 **[4]**, 22H-F_6-1,25$(OH)_2$-24-*ene*-VD_3 (mvd3400) **[5]**, 24H-F_6-1,25$(OH)_2$-22-*ene*-VD_3 (mvd1400) **[6]**, 24H-F_6-1,22(*S*),25$(OH)_3$-VD_3 (DD-003) **[7]**, 24H-F_6-1,22(*R*),25$(OH)_3$-VD_3 (DD-004) **[8]**, 24H-F_6-1,22(*S*),25$(OH)_3$-24-*yne*-VD_3 (DD-005) **[9]**, 24H-F_6-1,22(*R*),25$(OH)_3$-24-*yne*-VD_3 (mvd-1235) **[10]**, 24H-F_6-1,25$(OH)_2$-22-oxa-VD_3 (DD-006) **[11]**, 1-hydroxy-22-*ene*-cyclohexyl VD_3 (DD-007) **[12]**, F_6-1,25$(OH)_2$-22-*ene*-VD_3 (DD-008) **[13]**, F_6-1,25$(OH)_2$-22-*ene*-VD_3 (DD-009) **[14]**, 24H-F_6-1,24(*R*),25$(OH)_3$-VD_3 (DD-010) **[15]**, 24H-F_6-1,24(*S*),25$(OH)_3$-VD_3 (DD-011) **[16]**, 24H-F_6-1,23(*R*),25$(OH)_3$-VD_3 (DD-014) **[17]** and 24H-F_6-1,23(*S*),25$(OH)_3$-VD_3 (DD-015) **[18]** were synthesized by DAIKIN industry, Ibaragi, Japan and their structures are shown in Figure 1.

Cell culture. Human promyelocytic leukemic HL-60 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere. Viable cell number was determined by trypan blue exclusion, under light microscopy.

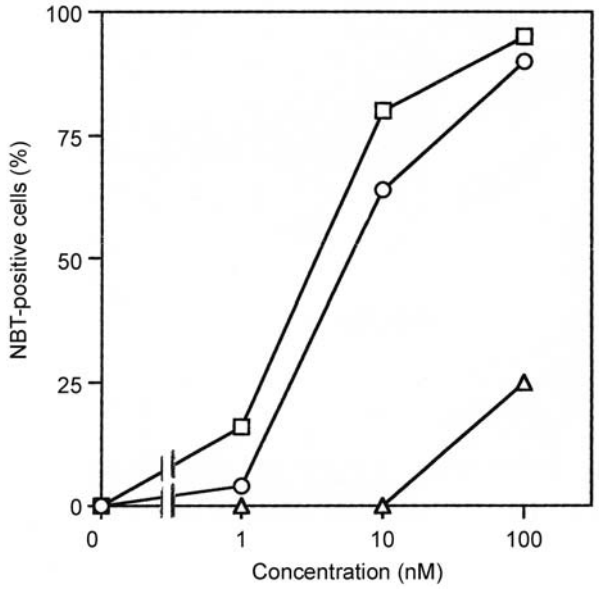

Figure 2. *Induction of NBT-reducing activity in HL-60 cells by hexafluorotrihydroxyvitamin D_3 derivatives. HL-60 cells were incubated for 3 days with the indicated concentrations of 1,25(OH)$_2$D$_3$ [1](○), DD-003 [7](□) or DD-004 [8](△), and the percent of NBT-positive cells was determined. At least 200 cells in each preparation were counted by light microscopy. Each value represents mean from two determinations.*

Table I. *Relationship between differentiation-inducing activity and hypercalcemic activity of hexafluorotrihydroxyvitamin D_3 derivatives.*

Compounds	MW	Differentiation-inducing activity (EC_{50}: nM) [a]	Serum calcium increasing activity (% of **[1]**) [b]
1 1,25$(OH)_2VD_3$	416.64	6.0	100
2 $F_6$1,25$(OH)_2VD_3$	524.59	0.85	>100
3 $F_6$1,25$(OH)_2VD_3$C28	538.61	1.2	100
4 $F_6$1,25$(OH)_2VD_3$C29	552.64	8.0	31
5 MVD-3400	536.60	1.6	18
6 MVD-1400	536.60	1.5	>100
7 DD-003($C_{28}H_{40}O_4F_6$)	554.62	3.5	5
8 DD-004($C_{28}H_{40}O_4F_6$)	554.62	>100	<1
9 DD-005 ($C_{28}H_{36}O_4F_6$)	550.59	3.6	7
10 MVD-1235 ($C_{29}H_{36}O_4F6$)	550.63	4.5	6
11 DD-006 ($C_{28}H_{38}O_4F_6$)	540.59	0.55	15
12 DD-007 ($C_{29}H_{38}O_2F_6$)	532.61	>100	>100
13 DD-008 ($C_{28}H_{38}O_3F_6$)	536.60	6.0	>100
14 DD-009 ($C_{28}H_{38}O_3F_6$)	536.60	1.0	>100
15 DD-010 ($C_{28}H_{40}O_4F_6$)	554.62	0.58	>100
16 DD-011 ($C_{28}H_{40}O_4F_6$)	554.62	0.50	>100
17 DD-014 ($C_{28}H_{40}O_4F_6$)	554.62	2.7	25
18 DD-015 ($C_{28}H_{40}O_4F_6$)	554.62	1.4	<10

a) determined as described in Figure 2.
b) determined as described in Figure 4.

Induction of differentiation. HL-60 cells were inoculated onto a 96-microwell plate at the cell density of 3-4 x 10^4 cells/0.2 ml. Each sample (20 μl) was added and the cells were incubated for 3 days. One hundred μl of supernatant was removed and 100 μl of NBT reagent (2 mg/ml) containing 0.33 μg/ml TPA was added. After incubation at 37˚C for 40 minutes, the percent of NBT-positive cells was counted (8).

Determination of serum calcium concentration. Wistar rats (3 weeks old, male, Japan SLC, Co.) were fed for 18 days with low calcium, vitamin D-free diet to yield animals with a lower concentration of calcium in the serum. These rats (5 rats/group) were administered *s.c.* every day with 0.1 ml of vehicle (95% propylene glycol) or sample dissolved in the same solvent. The blood was collected from the tail vein every 24 hours thereafter and the calcium concentration was determined by calcium binding reagent (Sigma Co.).

Computational details. Theoretical calculations were carried out, using the restricted Hartree-Fock level (HPL) PM3 semiempirical method, as implemented in the MOPAC program on a Tektronix CAChe work system (version 3.8). The octanol-water partition coefficient (log P) was calculated, using a relative permittivity of 78.4 for water for the COSMO model. Giometries were optimized in internal coordinates and were terminated when the Herberts test was satisfied in the eigenvector following method (EP) (9-11).

Results

Differentiation-inducing activity. 1,25$(OH)_2D_3$ **[1]** induced the differentiation of HL-60 cells, which showed higher NBT-reducing activity (Figure 2) and lower proliferation activity (data not shown). From the dose-response curve, the concentration which produced 50% NBT-positive cells (EC_{50}) of compound **[1]** was calculated to be 6 nM. DD-003 **[7]** showed slightly higher activity (CC_{50}=3.5 nM), whereas DD-004 **[8]** was much less active (CC_{50} >100 nM) (Figure 2). Among a total of 18 hexafluorotrihydroxyvitamin D_3 derivatives, DD-011 **[16]** (CC_{50}=0.5 nM) showed the greatest differentiation-inducing activity, followed by DD-006 **[11]** (CC_{50}=0.55 nM) > DD-010 **[15]** (0.58 nM) > ($F_6$1,25$(OH)_2D_3$) F6VD3 **[2]** (0.85 nM) >DD-009 **[14]** (1.0 nM) > F6c28 **[3]** (1.2 nM) > DD-015 **[18]** (1.4 nM) > mvd1400 **[6]** (1.5 nM) > mvd3400 **[5]** (1.6 nM) > DD-014 **[17]** (2.7 nM) > DD-003 **[7]** (3.5 nM) > DD-005 **[9]** (3.6 nM) > mvd-1235 **[10]** (4.5 nM) > DD-008 **[13]** (6.0 nM) = **[1]** (Table I). These compounds also inhibited the growth of HL-60 cells, more potently than 1,25$(OH)_2D_3$ (data not shown). F61,25$(OH)_2D_3$C29 **[4]** was slightly less active (CC_{50}=8.0 nM). On the other hand, DD-004 **[8]**, an isomer of DD-003 **[7]**, showed much reduced activity (CC_{50}>100

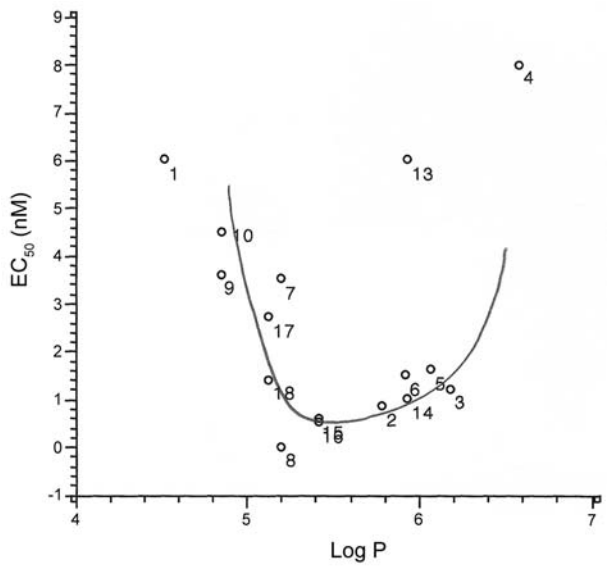

Figure 3. *Relationship between the differentiation-inducing activity and the octanol-water partition coefficient (log P). Data of* ***[8]*** *and* ***[12]*** *were deleted, due to their inactivity (CC_{50}>100 nM). Data of* ***[11]*** *were deleted, due to its extensive insolubility in water.*

nM) (Figure 2), suggesting the importance of the conformation around the OH at C-22 of the side chain in determining the differentiation-inducing activity.

When the differentiation-inducing activity (EC_{50}) of these compounds was plotted as a function of the octanol-water partition coefficient (log P) used as a parameter of hydrophobicity, a bell-shaped curve was produced (Figure 3). The maximum differentiation-inducing activity was found between the log *P* values of 5.4 to 5.8.

Calcium-increasing activity. $1,25(OH)_2D_3$ **[1]** dose-dependently increased the serum calcium concentration (Figure 4A). From the dose-response curve (Figure 4B), the calcium-increasing activity of DD-003 **[7]** was found to be 20-fold less, as compared with that of $1,25(OH)_2D_3$ **[1]** (Table I). The hypercalcemic activity (serum calcium-increasing activity) of other derivatives was similarly determined from the dose-response curve and expressed as % of that of $1,25(OH)_2D_3$ **[1]** (Table I). The compound which showed the lowest hypercalcemic activity was DD-004 **[8]** (<1%), followed by DD-003 **[7]** (5%) < mvd1235 **[10]** (6%) < DD-005 **[9]** (7%) < DD-015 **[18]** (<10%) < DD-006 **[11]** (15%) < mvd3400 [5] 18%) < DD-014 **[17]** (25%) < F6C29 **[4]** (31%).

On the other hand, compounds DD-007 **[12]**, DD-008 **[13]**, DD-011 **[16]**, DD-009 **[14]** and F_6D_3 **[2]** showed higher hypercalcemic activity than $1,25(OH)_2D_3$ **[1]**(Table I). When the differentiation-inducing activity of fluorinated vitamin D_3 derivatives was plotted as a function of

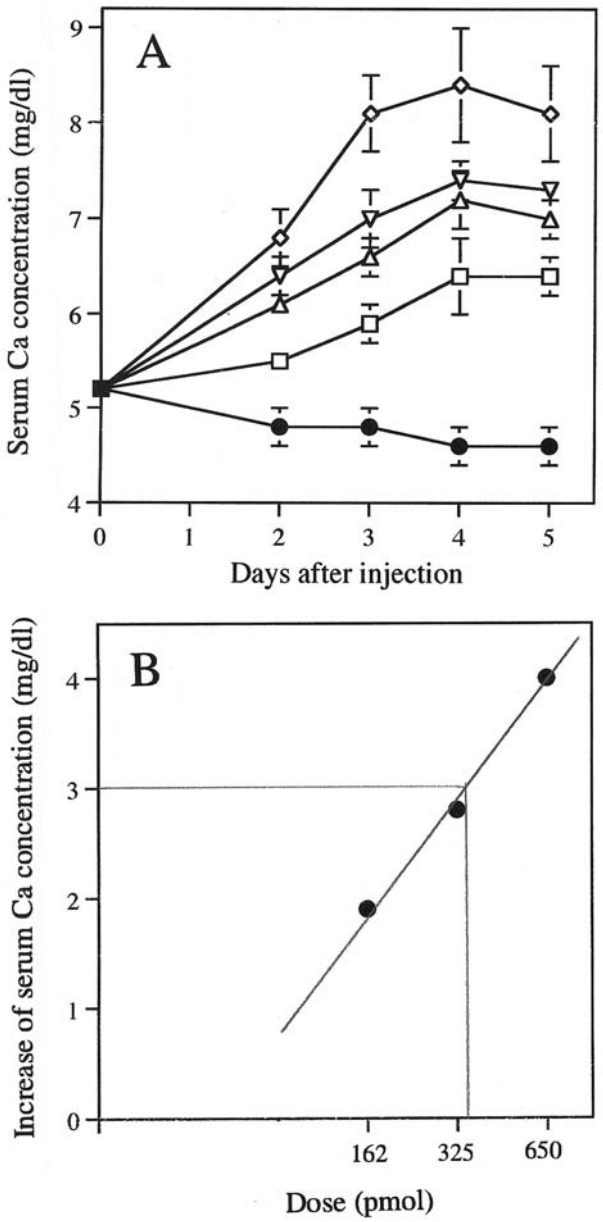

Figure 4. *(A) Hypercalcemic activity of $1,25(OH)_2D_3$* ***[1]*** *and DD-003* ***[7]****. Rats were administered s.c. with vehicle (●) or 162.5 (▽), 325 (△) or 650 pmol (◇) of $1,25(OH)_2D_3$* ***[1]*** *or 4 μg (7168 pmol) of DD-003* ***[7]*** *(▽), and the serum calcium concentration was determined. (B) Dose-response curve of the increase of serum calcium concentration.*

hypercalcemic activity, there was no clear-cut relationship between these two parameters (Figure 5).

In vivo antitumor activity. Pretreatment of mice three times (at -5, -3 and -1 day) with *s.c.* DD-003 **[7]** (4 μg/kg) significantly reduced the growth rate of human colon cancer HT-29 transplanted into the back of the mice (Figure 6).

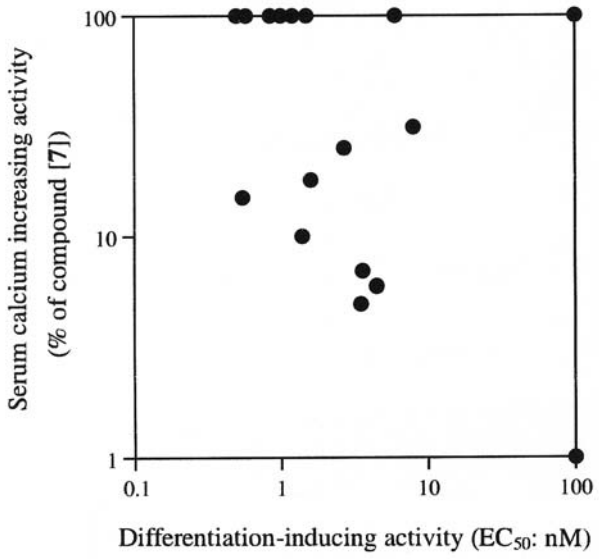

Figure 5. *Relationship between the differentiation-inducing activity and hypercalcemic activity. These data are derived from Table I.*

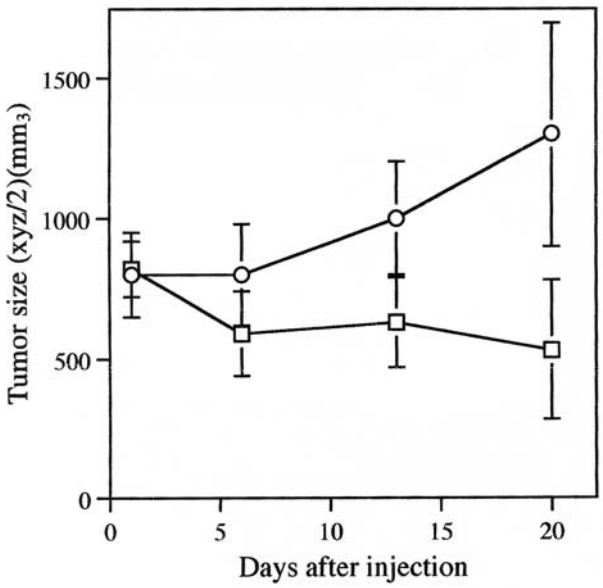

Figure 6. *Inhibition of HT-29 cell growth by intratumor administration of DD-003. Nude mice were inoculated with 1 x 10^6 HT cells. When the tumor size reached 1 cm diameter, the mice were administered with vehicle (●)or DD-003 [7](4 μg/kg)(□) at the indicated times and the tumor size (xyz/2) was determined.*

Discussion

The present study demonstrates that the differentiation-inducing activity of $1,25(OH)_2D_3$ was increased by the introduction of 6 fluorides, in agreement with previous publications (12). Among these derivatives, compounds **[2, 3, 5-7, 9-11, 14-18]** showed higher differentiation-inducing activity against HL-60 cells, as compared with the activated form of vitamin D_3 **[1]**. Compound **[2]** showed higher differentiation-inducing activity, but also higher hypercalcemic activity. Introduction of the side chain to **[2]** (**[3], [4]**) reduced both the differentiation-inducing activity and hypercalcemic activity. Introduction of a double bond to the side chain of **[2]** (**[13, 14]**) reduced the differentiation-inducing activity, but increased the hypercalcemic activity. Introduction of a double bond to the side chain (C-22, C-23) of **[3]**(**[5, [6]**] slightly reduced the differentiation-inducing activity, but differently modulated the hypercalcemic activity, depending upon the position of the introduced double bond. It has been reported that the configuration of the double bond does not significantly affect the differentiation-inducing activity (12). Introduction of the OH group to C-22 of **[3]** (**[7, 8]**) reduced the differentiation-inducing activity and hypercalcemic activity. Introduction of the OH group to C-23 of compound 3 (**[17, 18]**) slightly reduced the differentiation-inducing activity and the hypercalcemic activity. Introduction of the OH group to C-25 of **[3]** (**[15, 16]**) slightly increased the differentiation-inducing activity and the hypercalcmic activity. Introduction of the O atom to the side chain (C-22) of **[3]** (**[11]**) elevated the differentiation-inducing activity and reduced the serum hypercalcemic activity, in agreement with a previous report that introduction of the O atom to C-22 of **[1]** reduced the calcium-elevating activity (13). Compound **[12]** showed very weak differentiation-inducing activity, but elevated the serum calcium concentration. This suggests that the differentiation-inducing activity of vitamin D_3 analogs does not always correlate with the hypercalcemic activity. Compound **[12]** may belong to the vitamin D_3 derivatives without differentiation-inducing activity, such as $1,25(OH)_2$.26,23-lactoneVD_3 analogs (3).

Compounds **[3, 7, 11, 17]** seem to be promising for cancer therapy, since they had relatively higher differentiation-inducing activity, with lesser hypercalcemic activity, as compared with **[1]**. The other favorable property of these derivatives is their *in vivo* antitumor activity. Intratumor or oral injection of or pretreatment with DD-003 **[7]** significantly reduced the tumor size of nude mouse transplanted with colon cancer. Oral administration of DD-003 **[7]** (40 μg/kg) significantly reduced the growth rate of human colon cancer HT-29 and significantly prolonged the survival time of mice transplanted with M1 mouse leukemic cells (data not shown). These results further confirm the antitumor potential of hexafluorotrihydroxyvitamin D_3

derivatives. To establish cancer therapy by differentiation induction, it is necessary to screen vitamin D_3 derivatives which have potent differentiation-inducing activity with lower hypercalcemic activity. The present study may provide the basis for the synthesis of such compounds.

We found that the differentiation-inducing activity of these compounds depended on their hydrophobicity (calculated by log P). However, the optimal log P value (5.4-5.8) was significantly higher than that of Eugenol-related compounds (9), vitamin K (10) and flavonoids (11). The shift to higher log P value may be the interaction between vitamin D_3 derivatives and vitamin D-binding proteins present in FBS, added as nutritional supplements to RPMI1640 medium. The semiempirical method provides information such as log P, Van der Wals radius and hydration energy. This method can be applied to estimate the relative potency of structurally-related compounds. We have recently applied this method for the estimation of the apoptosis-inducing activity of gallic acid and its related compounds (14) and other polyphenols (Ishihara *et al.*, in preparation).

Acknowledgements

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