Enhancement of Activity of 1α,25-Dihydroxyvitamin D_3 for Growth Inhibition and Differentiation Induction of Human Myelomonocytic Leukemia Cells by Tretinoin Tocoferil, an α-Tocopherol Ester of All-*trans* Retinoic Acid

By Makoto Makishima, Yasuhiro Kanatani, Yuri Yamamoto-Yamaguchi, and Yoshio Honma

Tretinoin tocoferil is an α-tocopherol ester of all-*trans* retinoic acid (RA) and safely used in the treatment of skin ulcer. Tretinoin tocoferil inhibited proliferation of human promyelocytic leukemia HL-60 cells and induced granulocytic differentiation of the cells, but less than RA. α-Tocopherol did not affect differentiation of HL-60 cells, but at high concentrations enhanced its nitroblue tetrazolium (NBT)-reducing activity and expression of surface antigen CD11b, which are markers of myelomonocytic differentiation induced by RA. Tretinoin tocoferil increased NBT reduction in HL-60 cells treated with RA. It also enhanced the differentiation of HL-60 cells induced by dimethyl sulfoxide, phorbol-12-myristate 13-acetate or 1α,25-dihydroxyvitamin D_3 (VD_3). In combination with a low concentration of VD_3, it induced the NBT-reducing activity of human monoblastic U937 cells very effectively. Moreover, it enhanced the differentiation of human myelomonocytic ML-1, THP-1, P39/TSU, and P31/FUJ cells induced by VD_3. In combination with VD_3, it synergistically inhibited the proliferation of HL-60, U937, ML-1, THP-1, P39/TSU, and P31/FUJ cells and decreased the effective concentration of VD_3 to a 10^{-10} mol/L level. Because tretinoin tocoferil was reported to induce neither retinoid-related toxicity nor teratogenicity, the therapeutic advantage of the use of it in treatment of myelomonocytic leukemia is suggested.

RETINOIDS ARE INVOLVED in the control of cell proliferation, cell differentiation, and embryonic development. They can induce the differentiation of several tumor cell lines including those derived from leukemia, melanoma, neuroblastoma, and epithelial cancer.[1] The administration of all-*trans* retinoic acid (RA); an active form of natural retinoid, was reported to induce complete remission in more than 90% of patients with acute promyelocytic leukemia with promyelocytic myeloid leukemia/retinoic acid receptor α (PML/RARα) gene rearrangement.[2] RA was less effective in clinical trials against other hematologic malignancies.[3] Administration of a high dose of RA induces many adverse effects in the skin, central nervous system, liver, and other organs, and these complications may become life-threatening in some patients.[4] On the other hand, continuous administration of RA induces binding proteins such as cellular RA-binding proteins (CRABPs) in many tissues, and the resulting decrease in the plasma concentration of RA leads to the relapse of leukemia.[5]

A number of synthetic retinoids have been developed, but their biologic activities were found to be associated with clinical disadvantages such as toxicity and teratogenicity.[6] The development of selective retinoids that affect specific types of retinoid receptors is one approach to overcome these adverse effects.[6,7] For example, Am80, a retinobenzoic acid, binds to RAR-α and RAR-β, but not RAR-γ, and Ch55, another retinobenzoic acid, binds to RARs, but not CRABP. Both retinoids were more potent than RA in some cells, but the effectiveness was not sufficient in the other cells.[6,8] Another approach is the use of retinoids in combination with other drugs. Retinoids in combinations with interferon, butyrate or 1α,25-dihydroxyvitamin D_3 (VD_3) were reported to be more effective for inducing differentiation of leukemia cells than these agents singly.[9-12] The clinical usefulness of these combinations in the treatment of leukemia, however, has not been reported.

α-Tocopherol is another essential fat-soluble vitamin, vitamin E, and has biological activities such as antioxidant property and shows anticancer and immunostimulatory effects.[13] In vitro studies showed that α-tocopherol acid succinate inhibited growth and/or induced morphological differentiation in several types of malignancies including leukemia.[14,15] α-Tocopherol reduced the carcinogenicity of chemical carcinogens in animal models,[16,17] and epidemiological studies showed that dietary vitamin E reduced the risk of esophageal and gastric cancers.[18,19]

Tretinoin tocoferil is a unique compound, an α-tocopherol ester of RA (Fig 1). It is used safely in the treatment of skin ulcers in Japan.[20] Because both RA and α-tocopherol show an anticancer effect, the activity of tretinoin tocoferil on leukemia is very interesting. In this report, we examined the effects of this unique retinoid analog on proliferation and differentiation of human myelomonocytic leukemia cells.

MATERIALS AND METHODS

Materials. Tretinoin tocoferil (tocoretinate/(±)-3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoate) (Fig 1) was synthesized by Pharmaceutical Research Center, Nisshin Flour Milling Co (Saitama, Japan) and gifted from Lederle (Saitama, Japan). Tretinoin tocoferil was dissolved in liquid paraffin at 0.352 mol/L and diluted with ethanol to 4×10^{-2} mol/L. Dimethyl sulfoxide (DMSO) and VD_3 were purchased from Wako Pure Chemical Industry (Osaka, Japan), and nitroblue tetrazolium (NBT), RA, α-tocopherol, and phorbol-12-my-

From the Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Saitama, Japan.

Submitted February 14, 1995; accepted November 27, 1995.

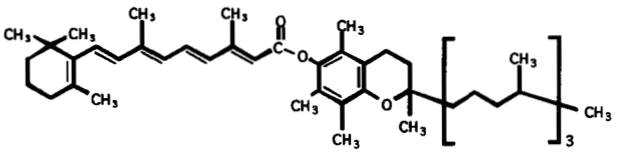
Supported in part by grants for Cancer Research from the Ministry of Education, Science, and Culture and a Grant for a Comprehensive 10-year Strategy for Cancer Control, Japan, from the Ministry of Health and Welfare.

Address reprint requests to Yoshio Honma, PhD, Department of Chemotherapy, Saitama Cancer Center Research Institute, 818 Komuro, Saitama 362, Japan.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

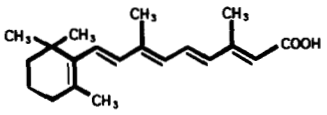
0006-4971/96/8708-0038$3.00/0

A



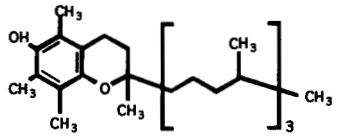
Tretinoin tocoferil

B



All-*trans* retinoic acid (tretinoin)

C



α-Tocopherol

Fig 1. Chemical structures of tretinoin tocoferil (A), all-*trans* retinoic acid (RA) (B), and α-tocopherol (C). RA is also named tretinoin.

ristate 13-acetate (TPA) were from Sigma (St Louis, MO). The concentrations of RA, α-tocopherol, and VD_3 in the ethanol stock solution were 4×10^{-3} mol/L, 6×10^{-2} mol/L, and 1.2×10^{-3} mol/L, respectively.

Cell lines and cell cultures. Human myeloid leukemia HL-60,[21] U937,[22] ML-1,[23] THP-1,[24] P39/TSU,[25] and P31/FUJ cells[26] were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 80 μg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air. P39/TSU cells were established from a patient with overt leukemia following the myelodysplastic syndrome,[25] and P31/FUJ cells were from a patient with acute monoblastic leukemia.[26] Both cell lines gave positive reactions for NaF-sensitive α-naphthyl butyrate esterase activity, Fcγ-receptors, C3-receptors, and showed phagocytic activity and reactivity with monoclonal antibodies characteristic of monocytic cells.[25,26] P39/TSU and P31/FUJ cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Cell growth and differentiation. Suspensions of cells were cultured with or without compounds in multidishes. Cell numbers were counted in a Model ZM Coulter Counter (Coulter Electronics, Luton, England). Cell viability was determined by exclusion of trypan blue. Cell morphology was examined in cell smears stained with May-Grünwald and Giemsa solutions by examination of more than 200 cells. NBT reduction was assayed microscopically and colorimetrically as reported previously.[27,28] In the microscopic assay, the percentage of cells staining intracellular blue-black formazan deposits was determined by examination of more than 200 cells. In the colorimetric assay, the reaction was stopped by adding HCl. The formazan deposits were solubilized by adding DMSO, and the absorption of the formazan solution at 560 nm was measured in a spectrophotometer (U-2000; Hitachi, Tokyo, Japan). Lysozyme activity in conditioned medium was determined by a method with lysoplates containing 1% agar, 1/15 mol/L sodium phosphate buffer (pH 6.6), 50 mmol/L NaCl, and heat-killed *Micrococcus lysodeikticus* (0.5 mg/mL).[27] One unit is equivalent to 1 μg/mL egg-white lysozyme. Expression of the granulocyte- and macrophage-specific antigen CD11b on the cell surface was determined by indirect immunofluorescent staining and flow cytometry.[28] The cells were incubated with mouse anti-CD11b antibody (Nichirei, Tokyo) and stained with fluorescein isothiocyanate (FITC) conjugated antimouse IgG (Tago, Burlingame, CA). The CD11b-positive cells were counted with a flow cytometer (Epics XL; Coulter Electronics).

Analysis of the effects of combinations of drugs. Isobologram analysis was used to determine the effects of combinations of drugs on leukemia cells.[29] Dose-dependent effects were determined for each compound and for one compound with fixed concentrations of another. The interaction of the two compounds was quantified by determining the combination index (CI) according to the classic isobologram equation,[30]

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

where Dx is the dose of one drug alone required to produce an effect and $(D)_1$ and $(D)_2$ are the doses of compounds 1 and 2, respectively, in combination that produce the same effect. From this analysis the combined effects of the two drugs can be assessed as: summation (additive or zero interaction) indicated as CI = 1, synergism indicated as CI < 1, or antagonism indicated as CI > 1.

Statistical evaluation. Statistical analyses in the experiments were performed using Student's *t*-test.

RESULTS

Effects of tretinoin tocoferil on growth and differentiation of human promyelocytic leukemia HL-60 cells. Human promyelocytic leukemia HL-60 cells are known to be induced to differentiate by several compounds including RA.[31] We examined the effect of tretinoin tocoferil on growth and differentiation of HL-60 cells. Tretinoin tocoferil inhibited proliferation of these cells concentration-dependently, its concentration for 50% growth inhibition (IC_{50}) being 1.3×10^{-4} mol/L (Fig 2A). However, the effective concentrations for tretinoin tocoferil for inhibiting cell growth were higher than those of RA (Fig 2B). Both RA and tretinoin tocoferil induced NBT-reducing activity, a typical marker of myelomonocytic differentiation,[31] of the HL-60 cells concentration-dependently (Table 1, Fig 2). In the cells treated with tretinoin tocoferil, however, the intensity of NBT-reducing activity was weak, and the colorimetric assay of NBT reduction, which was measured after dissolving the formazan deposits in the cells, showed low absorbance values as compared with those in the RA-treated cells (Fig 2C). Tretinoin tocoferil, like RA, induced morphological change of HL-60 cells into granulocytic lineage and increased the percentage of cells positive for CD11b antigen, which is a myeloid differentiation marker (Table 1). Although the culture medium treated with tretinoin tocoferil contains liquid paraffin and ethanol at the maximum concentrations of 0.0426% and 0.5%, respectively, the combination of liquid paraffin (0.0426%) and ethanol (0.5%) did not induce either NBT-reducing activity (Fig 2A) or morphological change of HL-

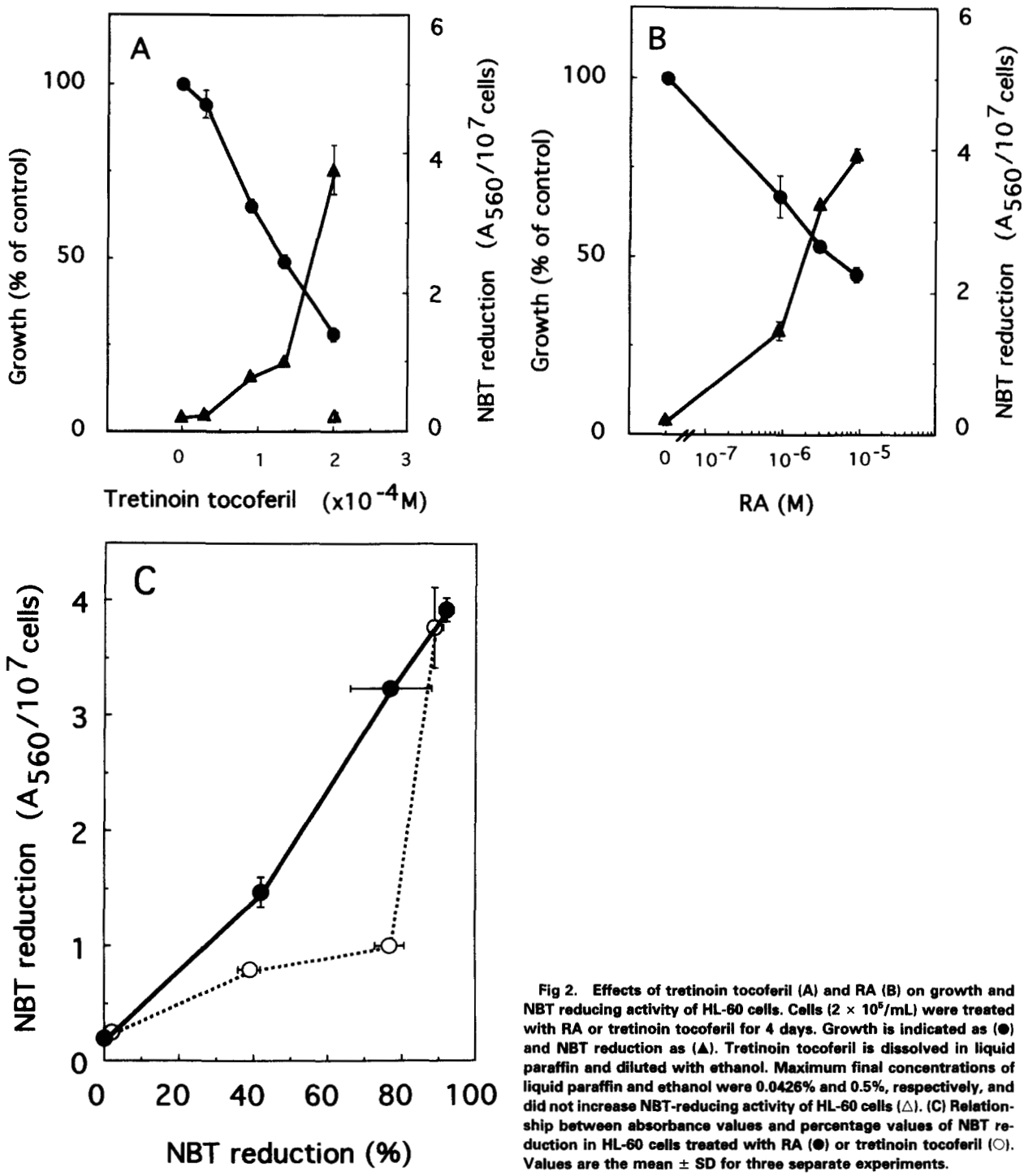


Fig 2. Effects of tretinoin tocoferil (A) and RA (B) on growth and NBT reducing activity of HL-60 cells. Cells (2×10^5/mL) were treated with RA or tretinoin tocoferil for 4 days. Growth is indicated as (●) and NBT reduction as (▲). Tretinoin tocoferil is dissolved in liquid paraffin and diluted with ethanol. Maximum final concentrations of liquid paraffin and ethanol were 0.0426% and 0.5%, respectively, and did not increase NBT-reducing activity of HL-60 cells (△). (C) Relationship between absorbance values and percentage values of NBT reduction in HL-60 cells treated with RA (●) or tretinoin tocoferil (○). Values are the mean ± SD for three separate experiments.

60 cells (data not shown). Thus tretinoin tocoferil was a weak inducer of granulocytic differentiation of HL-60 cells.

We found previously that α-tocopherol inhibited the differentiation of mouse myeloid leukemia M1 cells induced by dexamethasone.[32] Because tretinoin tocoferil is an α-tocopherol ester of RA, we examined whether α-tocopherol affected the differentiation of HL-60 cells induced by RA. α-Tocopherol at concentrations of up to 3×10^{-4} mol/L neither inhibited proliferation nor induced NBT-reducing activity of the HL-60 cells, and it only slightly affected the growth inhibition induced by RA (Fig 3). On the other hand, it significantly enhanced the induction of NBT-reducing ac-

Table 1. Effects of Tretinoin Tocoferil on Differentiation of HL-60 Cells

Treatment	NBT-Reducing Cells (%)	Morphological Changes (%)			CD11b-Positive Cells (%)	Viable Cells (%)
		Pro	Mye	Gr		
None	0 ± 0	100	0	0	0 ± 0	96 ± 0
TT 1.35×10^{-4} mol/L	77 ± 4	33	41	26	19 ± 1	99 ± 1
2×10^{-4} mol/L	89 ± 2	12	36	52	41 ± 11	98 ± 0
RA 9×10^{-6} mol/L	92 ± 2	1	25	74	23 ± 2	97 ± 1

Cells (1 to 2×10^5 cells/mL) were cultured with TT or RA for 4 days (for assay of NBT reduction, CD11b expression and viability) and 6 days (for assay of morphological changes). Values are means (±SD) for three separate experiments.

Abbreviations: TT, tretinoin tocoferil; Pro, promyelocytes; Mye, myelocytes; Gr, granulocytes.

tivity and expression of CD11b surface antigen of the cells induced by RA (Fig 3, Table 2).

Next we examined the effect of tretinoin tocoferil on the induction of NBT-reducing activity of HL-60 cells by RA. As shown in Fig 4, the addition of tretinoin tocoferil with RA significantly enhanced the NBT-reducing activity of HL-60 cells: tretinoin tocoferil at 9×10^{-5} mol/L increased NBT reduction in HL-60 cells treated with 9×10^{-7} mol/L and 3×10^{-6} mol/L RA 2.9-fold ($P < .0001$) and 2.0-fold ($P < .001$); respectively. Tretinoin tocoferil and RA additionally increased CD11b-positive HL-60 cells (Table 2). Thus the effect of RA on differentiation was enhanced by tretinoin tocoferil.

Effects of the combinations of tretinoin tocoferil and differentiation inducers on proliferation and differentiation of HL-60 cells. Next, we examined the effects of tretinoin tocoferil in combination with various differentiation inducers (Fig 5). DMSO is an inducer of granulocytic differentiation of HL-60 cells, while TPA and VD_3 are inducers of monocytic differentiation.[31] As shown in Fig 5A, DMSO induced NBT-reducing activity in HL-60 cells concentration-dependently, and tretinoin tocoferil enhanced this activity: DMSO at 1% and 1.2% increased the NBT-reducing activity to 1.6 ± 0.4 ($P < .05$) and 4.3 ± 0.3 $A_{560}/10^7$ cells ($P < .0001$), respectively, from 0.4 ± 0.0 $A_{560}/10^7$ cells in untreated cells, and tretinoin tocoferil enhanced the increase to 3.4 ± 0.3 (P

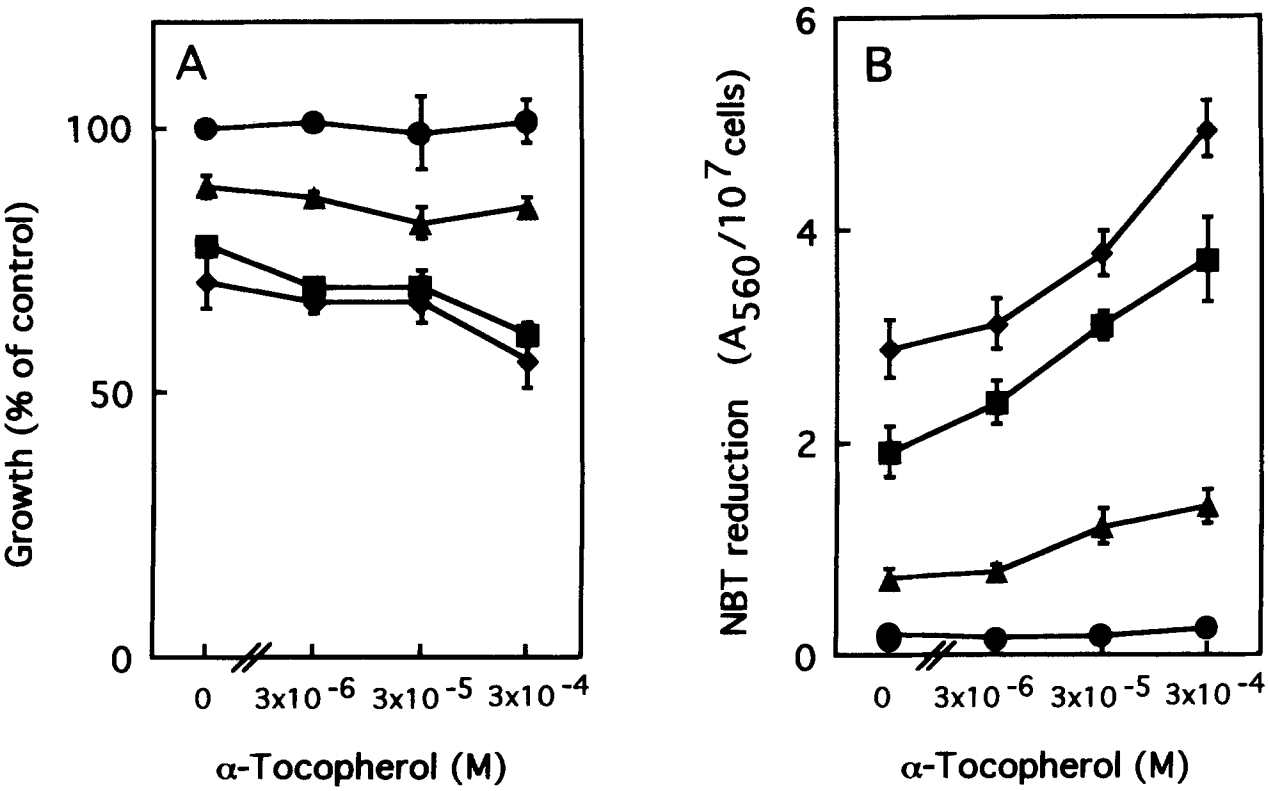


Fig 3. Effects of α-tocopherol on growth inhibition (A) and NBT-reducing activity (B) of HL-60 cells in combination with RA. Cells (2×10^5/mL) were treated with α-tocopherol in combination with 0 (●), 3×10^{-7} (▲), 9×10^{-7} (■), and 3×10^{-6} mol/L RA (◆) for 4 days. Values are the mean ± SD for three separate experiments.

Table 2. Effect of α-Tocopherol and Tretinoin Tocoferil on Differentiation of HL-60 Cells Induced by RA

Treatment	NBT-Reducing Cells (%)		CD11b-Positive Cells (%)		Viability (%)	
	−RA	+RA	−RA	+RA	−RA	+RA
None	0 ± 0	42 ± 1	0 ± 0	20 ± 3	94 ± 1	97 ± 1
α-Tocopherol 3×10^{-4} mol/L	0 ± 0	58 ± 5	0 ± 0	51 ± 6	92 ± 1	97 ± 0
TT 3×10^{-5} mol/L	2 ± 1	69 ± 1	8 ± 1	29 ± 2	98 ± 1	96 ± 0

Cells (2×10^5 cells/mL) were cultured with α-tocopherol or TT without or with 9×10^{-7} mol/L RA for 4 days. Values are the mean ± SD for three separate experiments.

< .005) and 5.4 ± 0.2 $A_{560}/10^7$ cells ($P < .01$), respectively. TPA less than 1 nmol/L induced NBT-reducing activity of HL-60 cells.[33] Tretinoin tocoferil also enhanced differentiation of HL-60 cells treated with TPA: TPA at 0.32 nmol/L increased NBT reduction to 3.4 ± 0.4 $A_{560}/10^7$ cells and tretinoin tocoferil further increased this activity to 6.5 ± 1.0 $A_{560}/10^7$ cells ($P < .01$; Fig 5B). The NBT-reducing activity of HL-60 cells induced by VD_3 was increased on combined treatment with tretinoin tocoferil: VD_3 (3×10^{-8} mol/L) plus tretinoin tocoferil (3×10^{-7} mol/L) increased the activity to 5.6 ± 1.1 $A_{560}/10^7$ cells, which was more than twice that with VD_3 alone (2.6 ± 0.3 $A_{560}/10^7$ cells) ($P < .02$) (Fig 5C). Lysozyme activity, another marker of myelomonocytic differentiation, of HL-60 cells was induced concentration-dependently by TPA and VD_3, and tretinoin tocoferil also enhanced this activity (Fig 5). The combination of liquid paraffin at 0.00006% and ethanol at 0.1%, which were solvents for 3×10^{-7} mol/L tretinoin tocoferil, did not increase either NBT-reducing (Fig 5 legend) or lysozyme activities of the cells treated with DMSO, TPA, or VD_3 (data not shown). Thus, tretinoin tocoferil enhanced the differentiation of HL-60 cells induced by DMSO, TPA, or VD_3.

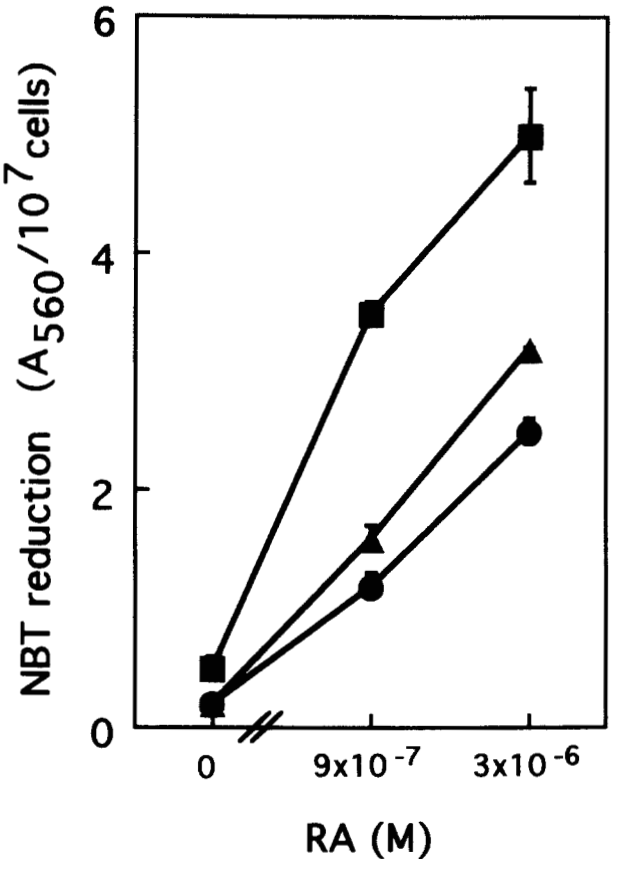


Fig 4. Effect of tretinoin tocoferil on NBT-reducing activity of HL-60 cells induced by RA. Cells (2×10^5/mL) were treated with 0 (●), 9×10^{-6} (▲), or 9×10^{-5} mol/L tretinoin tocoferil (■) for 4 days. Values are the mean ± SD for three separate experiments.

Synergistic effects of the combination of VD_3 and tretinoin tocoferil in inhibiting proliferation and inducing differentiation of monoblastic U937 cells. VD_3 is a promising inducer of differentiation for the treatment of some types of leukemia and cancer.[34] We next examined the effects of VD_3 plus tretinoin tocoferil on proliferation and differentiation of human monoblastic U937 cells. VD_3 inhibited proliferation of U937 cells concentration-dependently, its IC_{50} value after 6 days of treatment being 2.29×10^{-9} mol/L (Table 3). In combination with tretinoin tocoferil at a concentration that did not affect proliferation, VD_3 inhibited cell growth more effectively (Fig 6A). As shown in Table 3, 9×10^{-8} mol/L tretinoin tocoferil decreased the IC_{50} concentration of VD_3 to 4.26×10^{-10} mol/L (18.6% of that without tretinoin tocoferil). Figure 6B shows isoboles for the combination of VD_3 with tretinoin tocoferil that were isoeffective for inhibition of proliferation of U937 cells. These isoboles and the CI indices in Table 3 indicated that the combination of these drugs had synergistic effects. Tretinoin tocoferil at 4×10^{-9} mol/L to 4×10^{-7} mol/L did not induce NBT-reducing activity in U937 cells, but in combination with a low concentration of VD_3, this concentration range effectively induced the activity: VD_3 at 3×10^{-9} mol/L did not induce appreciable activity (0.5 ± 0.1 *v* 0.6 ± 0.0 $A_{560}/10^7$ cells for control cells), but in combination with 4×10^{-7} mol/L tretinoin tocoferil, it induced activity of 4.7 ± 0.5 $A_{560}/10^7$ cells ($P < .0005$; Fig 6C). Thus VD_3 and tretinoin tocoferil in combination showed synergistic effects in inhibiting proliferation and inducing differentiation of U937 cells.

Effects of VD_3 plus tretinoin tocoferil on proliferation and differentiation of several myelomonocytic leukemia cells. As shown in Fig 7, VD_3 induced NBT-reducing activity in human myelomonoblastic ML-1, monocytic THP-1, P39/TSU, and P31/FUJ cells concentration-dependently, and tretinoin tocoferil enhanced these activities: at 9×10^{-8} mol/L, it increased the activities of ML-1, THP-1, P39/TSU, and

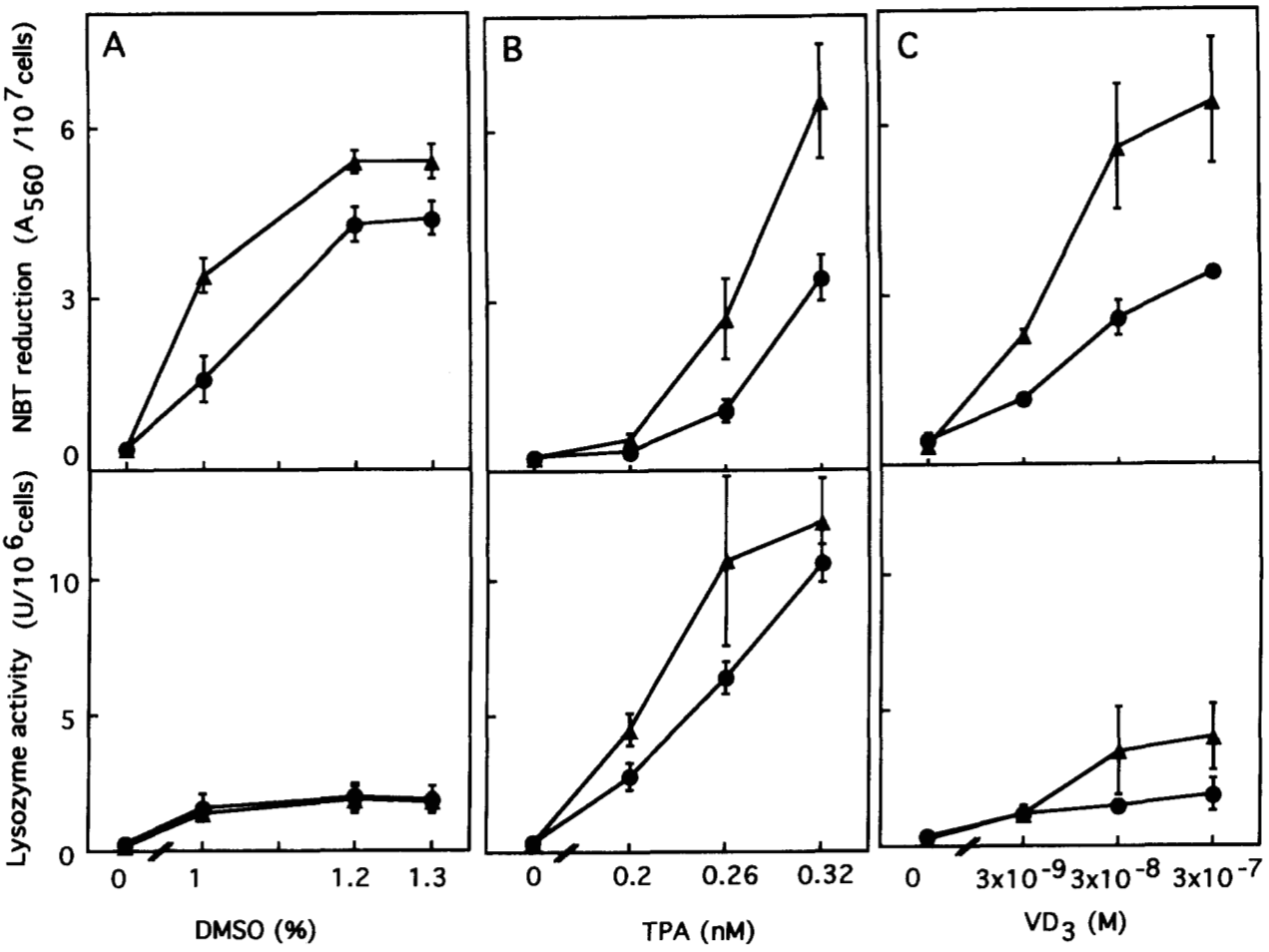


Fig 5. Effects of the combination of tretinoin tocoferil with DMSO (A), TPA (B), or VD_3 (C) on NBT-reducing and lysozyme activities of HL-60 cells. Cells (2×10^5 cells/mL) were treated with DMSO, TPA, or VD_3 in the absence (●) or presence of 3×10^{-7} mol/L tretinoin tocoferil (▲) for 4 days. The final concentrations of liquid paraffin and ethanol in the medium with tretinoin tocoferil were 0.00006% and 0.1%, respectively. The values of NBT reduction of the cells treated with the solvents (0.00006% liquid paraffin plus 0.1% ethanol) in combination with 1.3% DMSO, 0.32 mol/L TPA, and 3×10^{-7} mol/L VD_3 were 4.2 ± 0.3 versus 4.4 ± 0.3 $A_{560}/10^7$ cells for cells without the solvents, 3.8 ± 0.4 versus 3.4 ± 0.4 $A_{560}/10^7$ cells, and 3.9 ± 0.4 versus 3.4 ± 0.1 $A_{560}/10^7$ cells, respectively. The solvents did not effectively increase the lysozyme activities in the cells treated with TPA or VD_3 (data not shown). These findings indicated that the effect of tretinoin tocoferil was not due to either liquid paraffin or ethanol. Values are the mean ± SD for three separate experiments.

P31/FUJ cells induced by 3×10^{-8} mol/L VD_3 alone 1.9-fold ($P < .0001$), 2.1-fold ($P < .0005$), 1.7-fold ($P < .002$), and 1.3-fold ($P < .01$), respectively (Fig 7). Lysozyme activity was induced in P39/TSU and P31/FUJ cells by 3×10^{-8} mol/L VD_3 and tretinoin tocoferil at 9×10^{-8} mol/L slightly increased these activities (data not shown). Next, we examined the proliferations of these myelomonocytic leukemia cells on treatment with VD_3 plus tretinoin tocoferil for 6 days (Table 3). The IC_{50} concentrations of VD_3 for the myelomonocytic leukemia cells were decreased by combined treatment with tretinoin tocoferil and the CI values of all the cells we examined indicated synergism between VD_3 and tretinoin tocoferil in inhibiting proliferation. Addition of 9×10^{-8} mol/L tretinoin tocoferil decreased the IC_{50} concentration of VD_3 for inhibiting proliferation to 10^{-10} mol/L level in all the cells examined. Thus, tretinoin tocoferil and VD_3 enhanced differentiation and inhibited the proliferation of human myelomonocytic cells synergistically.

DISCUSSION

Tretinoin tocoferil is an α-tocopherol ester of RA, but its activity was different from those of RA and α-tocopherol. Tretinoin tocoferil at 1×10^{-8} mol/L and 1×10^{-7} mol/L enhanced migration of guinea pig peritoneal macrophage 1.4-fold and 1.8-fold, respectively, but RA or α-tocopherol at the same concentrations did not.[35] Because colchicine inhibited the induction of migration, tretinoin tocoferil was suggested to stimulate chemotaxis of macrophage.[35] Growth of human skin fibroblasts was enhanced 77% by treatment with 1×10^{-8} mol/L tretinoin tocoferil, but inhibited 61% by 5×10^{-10} mol/L RA, and not affected by 5×10^{-10} mol/L α-tocopherol.[36] Tretinoin tocoferil enhanced the differenti-

Table 3. Effects of Combination Treatment With Tretinoin Tocoferil and VD_3 on Proliferation of Human Myelomonocytic Leukemia Cells

Cell Line	IC_{50} of TT (mol/L)	IC_{50} of VD_3 (mol/L)		CI*
		−TT	+TT	
U937	1.05×10^{-6}	2.29×10^{-9}	4.26×10^{-10}	0.27
HL-60	6.26×10^{-6}	3.35×10^{-9}	5.32×10^{-10}	0.17
ML-1	6.61×10^{-7}	5.48×10^{-10}	2.42×10^{-10}	0.58
THP-1	9.22×10^{-7}	3.38×10^{-9}	7.79×10^{-10}	0.33
P39/TSU	1.17×10^{-6}	2.36×10^{-9}	7.04×10^{-10}	0.38
P31/FUJ	1.44×10^{-6}	1.48×10^{-9}	7.57×10^{-10}	0.57

Cells (2×10^4 cells/mL of U937, HL-60, THP-1, P39/TSU, and P31/FUJ cell lines and 4×10^4 cells/mL of ML-1 cell line) were cultured with test compounds for 6 days. IC_{50} values are concentrations of TT required for 50% inhibition of cell growth or concentrations of VD_3 required for 50% inhibition of cell growth in the absence or presence of TT (9×10^{-8} mol/L).

* Combination index at IC_{50}. CI values with a fixed concentration of TT (9×10^{-8} mol/L) were calculated as described in Materials and Methods. In the assay, CI = 1 indicates summation (additive or zero interaction), CI < 1 synergism, and CI > 1 antagonism.

ation of HL-60 cells induced by RA (Fig 4, Table 2), and the CI value of a combination of 9×10^{-8} mol/L tretinoin tocoferil and RA in growth inhibition of U937 cells after 6 days of treatment was 0.68, indicating synergism of these compounds (data not shown). Thus, tretinoin tocoferil has different biological activities from RA.

We previously reported that α-tocopherol inhibited differentiation of mouse myeloid leukemia M1 cells induced by dexamethasone.[32] Then, we examined whether α-tocopherol adversely affected differentiation of HL-60 cells. α-Tocopherol did not inhibit proliferation or induce the differentiation of HL-60 cells, but enhanced the differentiation induced by RA (Fig 3, Table 2). α-Tocopherol acid succinate was reported to induce differentiation of HL-60 cells and mouse melanoma cells.[14,15] On the other hand, Sokoloski et al[37] reported that α-tocopherol acid succinate alone did not induce differentiation of HL-60 cells, but it enhanced the differentiation induced by VD_3. Thus, α-tocopherol and its derivative have differentiation-enhancing activity on some cells. Although tretinoin tocoferil is an α-tocopherol ester of RA, it was reported to be stable.[38] Chromatogram obtained from the extracts of plasma, heart, liver, spleen, and bile of rats and dogs that received radio-labeled tretinoin tocoferil showed that 88% to 96% of it was unchanged and that RA was not detected.[38] Tretinoin tocoferil was not hydrolyzed by in vitro treatment with esterase.[38] These findings suggest that tretinoin tocoferil affects the growth and differentiation of leukemia cells without catabolizing to α-tocopherol and RA.

Retinoids, including RA, are reported to have toxicity and teratogenicity.[39,40] The LD_{50} concentration of RA on its intraperitoneal administration in rats is 158 mg/kg (data from Nippon Roche), whereas administration of more than 2,000 mg/kg tretinoin tocoferil, even intravenously, did not show any drug-related acute toxicity in rats.[39,41] Oral administration of RA at more than 15 mg/kg/d for 13 weeks had toxic effects on rats, but administration of tretinoin tocoferil at 300 mg/kg/d for 12 months did not have any chronic toxic effects.[39,42] The long-term treatment with tretinoin tocoferil at 300 mg/kg/d did not induce hypertriglycemia, which is commonly induced by active retinoids including RA and 13-cis retinoic acid.[40,42] In studies on reproductive function and fertility in rats, RA in oral administration of 5 mg/kg/d decreased fetal survival rate, but tretinoin tocoferil at 1,000 mg/kg/d did not show toxic effects for the survival, general signs, body weight, and food intake of parental animals; for the reproductive performance of parental animals; or for fetal growth and development.[39,43] In teratological studies in rabbits, oral administration of RA at 6 mg/kg/d induced malformations of fetuses, whereas tretinoin tocoferil at 1,000 mg/kg/d showed no drug-related effects on body weight, viability indexes or external, visceral or skeletal examinations in fetuses.[39,44] RA administered orally to rats at 5 mg/kg/d in perinatal and postnatal periods decreased viability of the fetus, but tretinoin tocoferil at 1,000 mg/kg/d did not show any adverse effects on either F1 or F2 offsprings.[39,45] Thus, tretinoin tocoferil is much less toxic than RA and does not induce any toxic or teratogenic effects even in high dose administrations. Combined treatment of U937 cells with 4×10^{-7} mol/L tretinoin tocoferil plus 3×10^{-9} mol/L VD_3, as shown in Fig 6C, induced NBT reduction to 4.7 ± 0.5 $A_{560}/10^7$ cells, while the treatment with 4×10^{-9} mol/L RA plus 3×10^{-9} mol/L VD_3 induced the reduction to the same level, 4.7 ± 0.3 $A_{560}/10^7$ cells (data not shown). Tretinoin tocoferil, when combined with VD_3, is 100 times less effective in inducing the differentiation of U937 cells than RA, but the teratological studies showed that tretinoin tocoferil is at least 167 to 200 times safer than RA.[43-45] These findings indicate that tretinoin tocoferil has more therapeutic potency than RA. Besa et al[46] reported that administration of α-tocopherol ameliorated the toxicity of 13-cis retinoic acid in a clinical trial to myelodysplastic syndrome. Therefore, α-tocopherol esterification of RA may contribute to reducing the toxicity without abolishing the differentiation-enhancing activity.

The pharmacologic study in patients with acute promyelocytic leukemia showed that the peak plasma concentration of RA following a single oral dose of RA at 45 mg/m^2 was 346 ± 266 ng/mL (1.15×10^{-6} mol/L), but the plasma concentrations declined to the physiologic level within 12 hours.[47] The short half-life of RA requires repeated administrations for achievement of effective concentrations. The continued administration of RA, however, increases catabolic enzyme cytochrome P450 and cellular binding protein such as CRABPII, and the resulting decrease of the plasma concentrations contributes to clinical resistance to RA.[5,47-49] On the other hand, when rats were treated with nontoxic 5 mg/kg tretinoin tocoferil intravenously and orally, its plasma concentrations were 10^{-7} mol/L and 10^{-8} mol/L, respectively, for 24 hours and 10^{-8} mol/L and 10^{-9} mol/L, respectively, even 7 days after administrations.[50] These concentrations of tretinoin tocoferil were effective for inhibition of growth and induction of differentiation of myelomonocytic leukemia cells in combination with other inducers such as VD_3 (Figs 5-7). Thus, addition of α-tocopheryl group to RA may contribute to the maintenance of its plasma concentrations in effective range.

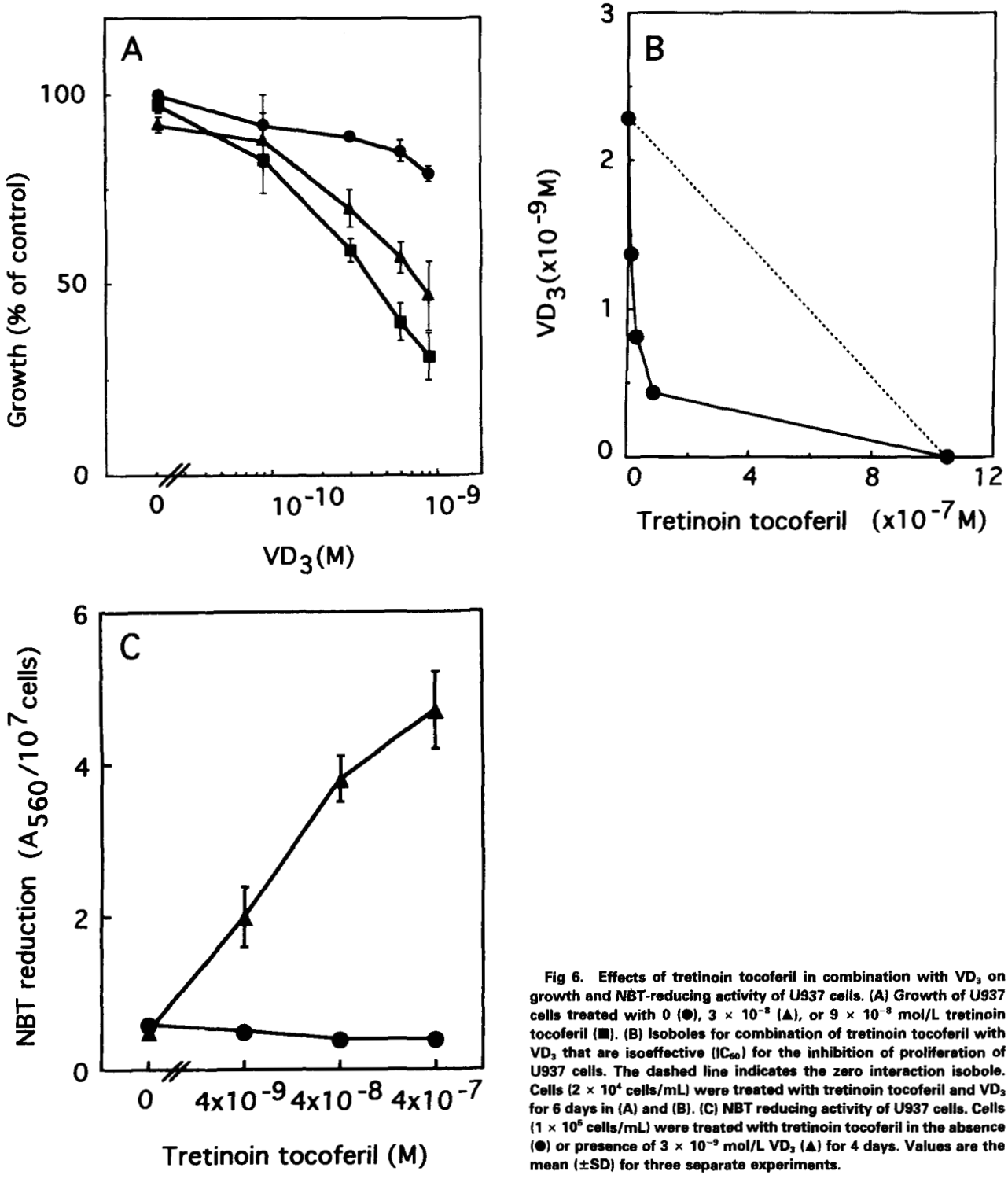


Fig 6. Effects of tretinoin tocoferil in combination with VD_3 on growth and NBT-reducing activity of U937 cells. (A) Growth of U937 cells treated with 0 (●), 3×10^{-8} (▲), or 9×10^{-8} mol/L tretinoin tocoferil (■). (B) Isoboles for combination of tretinoin tocoferil with VD_3 that are isoeffective (IC_{50}) for the inhibition of proliferation of U937 cells. The dashed line indicates the zero interaction isobole. Cells (2×10^4 cells/mL) were treated with tretinoin tocoferil and VD_3 for 6 days in (A) and (B). (C) NBT reducing activity of U937 cells. Cells (1×10^5 cells/mL) were treated with tretinoin tocoferil in the absence (●) or presence of 3×10^{-9} mol/L VD_3 (▲) for 4 days. Values are the mean (±SD) for three separate experiments.

VD_3 is a promising inducer for differentiation.[34] Although VD_3 is reported to induce differentiation of cell lines of leukemia, colon, and breast cancer, its adverse effects, mainly hypercalcemia, limit its clinical use in cancer treatment when its serum concentration exceeds 10^{-9} mol/L.[51] Several reports showed RA acted synergistically with VD_3 to induce differentiation of leukemia cells.[11,12] Similar to VD_3, however, RA was reported to cause hypercalcemia in the treatment of acute promyelocytic leukemia.[52-55] Hypercalcemia was also reported in clinical trials of another active

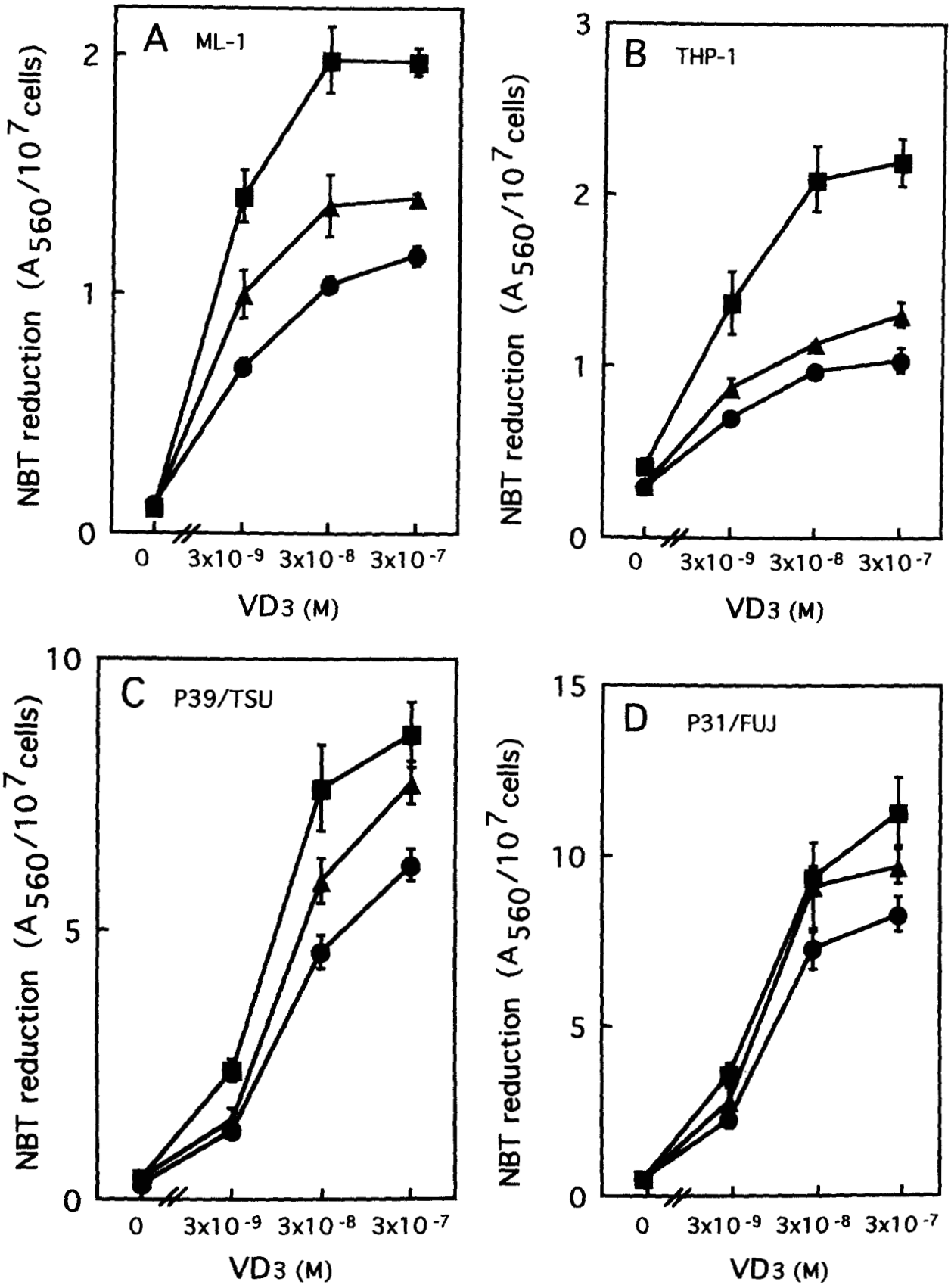


Fig 7. Effects of combination of tretinoin tocoferil with VD_3 on NBT-reducing activity of ML-1 (A), THP-1 (B), P39/TSU (C), and P31/FUJ cells (D). Cells (2×10^5 cells/mL) were treated with VD_3 and 0 (●), 9×10^{-9} (▲), or 9×10^{-8} mol/L tretinoin tocoferil (■) for 4 days. Values are the mean ± SD for three separate experiments.

retinoid, 13-cis retinoic acid.[56,57] Retinoids are known to induce skeletal abnormalities in hypervitaminosis A syndrome.[40] Therefore, the combination of a strong retinoid with VD_3 still has a risk of inducing hypercalcemia. On the contrary, tretinoin tocoferil in the long-term administration in rats did not induce hypercalcemia or any pathological changes of bone,[42] and its clinical toxicity in bone metabolism has not been reported. Thus, tretinoin tocoferil did not induce retinoid-related toxicity and teratogenicity. Therefore, as tretinoin tocoferil at the 10^{-8} mol/L level and VD_3 at the 10^{-10} mol/L level synergistically inhibited proliferation of human myelomonocytic leukemia cells, the combined treatment with VD_3 and tretinoin tocoferil may be useful in therapy of myelomonocytic leukemia.

REFERENCES

1. Warrell RP Jr: Applications for retinoids in cancer therapy. Semin Hematol 31:1, 1994 (suppl 5)
2. Warrell RP Jr, de The H, Wang Z-Y, Degos L: Acute promyelocytic leukemia. New Engl J Med 329:177, 1993
3. Tallman MS: All-*trans*-retinoic acid in acute promyelocytic leukemia and its potential in other hematologic malignancies. Semin Hematol 31:38, 1994 (suppl 5)
4. Frankel SR, Eardley A, Lauwers G, Weiss M, Warrell RP Jr: The "retinoic acid syndrome" in acute promyelocytic leukemia. Ann Intern Med 117:292, 1992
5. Warrell RP Jr: Retinoid resistance in acute promyelocytic leukemia: New mechanisms, strategies, and implications. Blood 82:1949, 1993
6. Hashimoto Y, Shudo K: Retinoids and their nuclear receptors. Cell Biol Rev 25:209, 1991
7. Dawson MI, Elstner E, Kizaki M, Chen D-L, Pakkala S, Kerner B, Koeffler HP: Myeloid differentiation mediated through retinoic acid receptor/retinoic X receptor (RXR) not RXR/RXR pathway. Blood 84:446, 1994
8. Takahashi N, Breitman TR: Induction of differentiation and covalent binding to proteins by the synthetic retinoids Ch55 and Am80. Arch Biochem Biophys 314:82, 1994
9. Peck R, Bollag W: Potentiation of retinoid-induced differentiation of HL-60 and U937 cell lines by cytokines. Eur J Cancer 27:53, 1991
10. Breitman TR, Chen Z-X, Takahashi N: Potential applications of cytodifferentiation therapy in hematologic malignancies. Semin Hematol 31:18, 1994 (suppl 5)
11. Brown G, Bunce CM, Rowlands DC, Williams GR: All-*trans*-retinoic acid and 1α,25-dihydroxyvitamin D_3 co-operate to promote differentiation of the human promyeloid leukemia cell line HL60 to monocytes. Leukemia 8:806, 1994
12. He RY, Breitman TR: Combinations of 24,24-difluoro-1α,25-dihydroxyvitamin D_3 and either retinoic acid, sodium butyrate, dimethyl sulfoxide, or hexamethylene bisacetamide synergistically induce monocytoid differentiation of the human myeloid leukemia cell line HL-60. Biomed Pharmacother 46:313, 1992 (abstr)
13. Meydani M: Vitamin E. Lancet 345:170, 1995
14. Turley JM, Sanders BG, Kline K: RRR-α-Tocopheryl succinate modulation of human promyelocytic leukemia (HL-60) cell proliferation and differentiation. Nutr Cancer 18:201, 1992
15. Prasad KN, Edward-Prasad J: Effects of tocopherol (vitamin E) acid succinate on morphological alterations growth inhibition in melanoma cells in culture. Cancer Res 42:550, 1982
16. Cook MG, McNamara P: Effect of dietary vitamin E on dimethylhydrazine-induced colonic tumors in mice. Cancer Res 40:1329, 1980
17. Perchellet J-P, Owen MD, Posey TD, Orten DK, Schneider BA: Inhibitory effects of glutathione level-raising agents and D-α-tocopherol on ornithine decarboxylase induction and mouse skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. Carcinogenesis 6:567, 1985
18. Blot WJ, Li J-Y, Taylor PR, Guo W, Dawsey S, Wang G-Q, Yang CS, Zheng S-F, Gail M, Li G-Y, Yu Y, Liu B-Q, Tangrea J, Sun Y-H, Liu F, Fraumeni JF Jr, Zhang Y-H, Li B: Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. J Natl Cancer Inst 85:1483, 1993
19. Taylor PR, Li B, Dawsey SM, Li J-Y, Yang CS, Guo W, Blot WJ, the Linxian Nutrition Intervention Trials Study Group: Prevention of esophageal cancer: The nutrition intervention trials in Linxian, China. Cancer Res 54:2029s, 1994 (suppl)
20. Yasuno Y, Kishimoto S, Yamanishi K, Konishi K, Oka F, Oonishi M, Kagami K, Oki M, Maeda M, Sasaki Y, Komori Y, Wakabayashi S, Miura H, Inoue Y, Miyashita A, Tamaoki K, Okuda R, Ryu F: Evaluation of the effects of L-300 ointment in the treatment of intractable skin ulcers. Rinsho-iyaku (in Japanese) 6:2481, 1990
21. Collins SJ, Gallo RC, Gallagher RE: Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature 270:347, 1977
22. Sundström C, Nilsson K: Establishment and characterization of a human histiocytic lymphoma cell line. Int J Cancer 17:565, 1976
23. Minowada J: Immunology of leukemic cells, in Grunz F, Henderson E (eds): Leukemia. New York, NY, Grune & Stratton, 1982, p 119
24. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K: Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 26:171, 1980
25. Nagai M, Seki S, Kitahara T, Abe T, Minato K, Watanabe S, Shimoyama M: A novel human myelomonocytoid cell line, P39/Tsugane, derived from overt leukemia following myelodysplastic syndrome. Jpn J Cancer Res (Gann) 75:1100, 1984
26. Hirose M, Minato K, Tobinai K, Shimoyama M, Watanabe S, Abe T: A novel monocytoid cultured cell line, P31/Fujioka, derived from acute monoblastic leukemia. Jpn J Cancer Res (Gann) 73:735, 1982
27. Makishima M, Honma Y, Hozumi M, Sampi K, Hattori M, Ishikawa I, Ogura H, Motoyoshi K: Effects of novel uracil analogs on proliferation and differentiation of human myeloid leukemia cells. Exp Hematol 20:879, 1992
28. Tomida M: Induction of differentiation of WEHI-3B D^+ leukemic cells transfected with differentiation-stimulating factor/leukemia inhibitory factor receptor cDNA. Blood 85:217, 1995
29. Berenbaum MC: What is synergy? Pharmacol Rev 41:93, 1989
30. Chen Z-X, Breitman TR: Tributyrin: A prodrug of butyric acid for potential clinical application in differentiation therapy. Cancer Res 54:3494, 1994
31. Hozumi M: Fundamentals of chemotherapy of myeloid leukemia by induction of leukemia cell differentiation. Adv Cancer Res 38:121, 1983
32. Takenaga K, Honma Y, Hozumi M: Inhibition of differentiation of mouse myeloid leukemia cells by phenolic antioxidants and α-tocopherol. Jpn J Cancer Res (Gann) 72:104, 1981
33. Okabe-Kado J, Honma Y, Kasukabe T, Hozumi M: Synthesis of active metabolite(s) from 1α-hydroxyvitamin D_3 by human monocytic leukemia cells. FEBS Lett 309:399, 1992

34. Bikle DD: Clinical counterpoint: Vitamin D: New actions, new analogs, new therapeutic potential. Endocrine Rev 13:765, 1992

35. Hamada H, Sakyo K, Tanaka H, Ogawa O, Nishiki K: Effect of tocoretinate on migration of cells. Pharmacometrics (in Japanese) 43:97, 1992

36. Sakyo K, Otsuka N, Hamada H, Nakaya N, Nakazawa T, Nakahara Y, Naruke T, Nishiki K, Ito A, Mori Y: Effect of tocoretinate on proliferation of normal human skin fibroblasts. Pharmacometrics (in Japanese) 43:103, 1992

37. Sokoloski JA, Hodnick WF, Mayne ST, Kim CS, Sartorelli AC: Induction of the differentiation of HL-60 leukemia cells by vitamin E and other antioxidants in combination with vitamin D_3. Proc Am Ass Cancer Res 36:348, 1995 (abstr)

38. Kuroda T, Nakazawa T, Watanabe T, Kawashima K, Arai I, Nakahara Y, Naruke T, Nishiki K, Inomata N: Metabolic fate of tocoretinate (3). Metabolism of ^{14}C-tocoretinate in rats and dogs. Pharmacometrics (in Japanese) 43:1, 1992

39. Kamm JJ: Toxicology, carcinogenicity, and teratogenicity of some orally administered retinoids. J Am Acad Dermatol 6:652, 1982

40. Lowe NJ, David M: New retinoids for dermatologic diseases. Uses and toxicity. Dermatol Clin 6:539, 1988

41. Harada Y, Narita H, Kashiwagi H, Misawa N, Takagi H, Takita S, Inomata N: Acute toxicity study of tocoretinate in mice, rats and dogs. Pharmacometrics (in Japanese) 43:7, 1992

42. Okazaki S, Mochizuki M, Masuda T, Takagi H, Takita S, Inomata N: A 12-month oral toxicity study of tocoretinate in rats. Pharmacometrics (in Japanese) 43:251, 1992

43. Ohta R, Hashimoto Y, Mizutani M, Misawa N, Inomata N: Reproductive and developmental toxicity study of tocoretinate (I). Effects on reproductive function and fertility in rats (segment I study). Pharmacometrics (in Japanese) 43:303, 1992

44. Tanaka N, Murakami Y, Noguchi O, Narita H, Hamada S, Ohashi M, Misawa N, Inomata N: Reproductive and developmental toxicity study of tocoretinate (III). Teratological study in rabbits by oral administration. Pharmacometrics (in Japanese) 43:323, 1992

45. Murakami Y, Ogasawara H, Sakauchi N, Narita H, Hamada S, Ohashi M, Noguchi O, Tanaka N, Misawa N, Inomata N: Reproductive and developmental toxicity study of tocoretinate (IV). Administration to female rats during the perinatal and postnatal periods. Pharmacometrics (in Japanese) 43:329, 1992

46. Besa EC, Abrahm JL, Bartholomew MJ, Hyzinski M, Nowell PC: Treatment with 13-cis-retinoic acid in transfusion-dependent patients with myelodysplastic syndrome and decreased toxicity with addition of alpha-tocopherol. Am J Med 89:739, 1990

47. Muindi JRF, Frankel SR, Huselton C, DeGrazia F, Garland WA, Young CW, Warrell RP Jr: Clinical pharmacology of oral all-*trans* retinoic acid in patients with acute promyelocytic leukemia. Cancer Res 52:2138, 1992

48. Cornic M, Delva L, Guidez F, Balitrand N, Degos L, Chomienne C: Induction of retinoic acid-binding protein in normal and malignant human myeloid cells by retinoic acid in acute promyelocytic leukemia patients. Cancer Res 52:3329, 1992

49. Rigas JR, Francis PA, Muindi JRF, Kris MG, Huselton C, DeGrazia F, Orazem JP, Young CW, Warrell RP Jr: Constitutive variability in the pharmacokinetics of the natural retinoid, all-*trans*-retinoic acid, and its modulation by ketoconazole. J Natl Cancer Inst 85:1921, 1993

50. Nakazawa T, Kuroda T, Arai I, Nakahara Y, Naruke T, Nishiki K, Inomata N, Tahara Y, Yanai M, Sakuma S, Kimura T: Metabolic fate of tocoretinate (1). Absorption and excretion of ^{14}C-tocoretinate in rats and dogs. Pharmacometrics (in Japanese) 43:205, 1992

51. Koeffler HP, Hiuji K, Itri L, the Southern California Leukemia Group: 1,25-Dihydroxyvitamin D_3: In vivo and in vitro effects on human preleukemic and leukemic cells. Cancer Treat Rep 69:1399, 1985

52. Akiyama H, Nakamura N, Nagasaka S, Sakamaki H, Onozawa Y: Hypercalcaemia due to all-*trans* retinoic acid. Lancet 339:308, 1992

53. Sakakibara M, Ichikawa M, Amano Y, Matsuzawa S, Agematsu K, Mori T, Koike K, Nakahata T, Komiyama A: Hypercalcemia associated with all-trans-retinoic acid in the treatment of acute promyelocytic leukemia. Leuk Res 17:441, 1993

54. Suzumiya J, Asahara F, Katakami H, Kimura N, Hisano S, Okumura M, Ohno R: Hypercalcaemia caused by all-trans retinoic acid treatment of acute promyelocytic leukaemia: Case report. Eur J Haematol 53:126, 1994

55. Lemež P: Hypercalcaemia caused by all-*trans* retinoic acid (ATRA) treatment in a case of acute promyelocytic leukaemia was manageable after decreasing the ATRA dose to 27 mg/m^2/day. Eur J Haematol 55:275, 1995

56. Cassidy J, Lippman M, Lacroix A, Peck G: Phase II trial of 13-cis-retinoic acid in metastatic breast cancer. Eur J Cancer Clin Oncol 18:925, 1982

57. Villablanca JG, Khan AA, Avramis VI, Reynolds CP: Hypercalcemia: A dose-limiting toxicity associated with 13-cis-retinoic acid. Am J Pediatr Hematol Oncol 15:410, 1993

1996 87: 3384-3394

Enhancement of activity of 1alpha, 25-dihydroxyvitamin D3 for growth inhibition and differentiation induction of human myelomonocytic leukemia cells by tretinoin tocoferil, an alpha-tocopherol ester of all-trans retinoic acid

M Makishima, Y Kanatani, Y Yamamoto-Yamaguchi and Y Honma

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.