Enhancement of Activity of 1α,25-Dihydroxyvitamin D₃ 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 D₃ (VD₃). In combination with a low concentration of VD₃, 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/PUJ cells induced by VD₃. In combination with VD₃, it synergistically inhibited the proliferation of HL-60, U937, ML-1, THP-1, P39/TSU, and P31/PUJ cells and decreased the effective concentration of VD₃ 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. © 1996 by The American Society of Hematology.

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. 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. RA was less effective in clinical trials against other hematologic malignancies. 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. 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.

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. The development of selective retinoids that affect specific types of retinoid receptors is one approach to overcome these adverse effects.

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. Because both RA and α-tocopherol show an antitumor 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 (tococretinate(+)−3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,-12-trimethyleneducl-2H-1-benzopyran-6-yl) (2F,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexene-1-yl)-2,4,6,8-nonatetraenoate) (Fig 1) was synthesized by Pharmaceutical Research Center, Nissin 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 VD₃ were purchased from Wako Pure Chemical Industry (Osaka, Japan), and nitroblue tetrazolium (NBT), RA, α-tocopherol, and phorbol-12-myristate 13-acetate or 1α,25-dihydroxyvitamin D₃ (VD₃) were reported to induce neither retinoid-related toxicity nor teratogenicity, the therapeutic advantage of the use of it in treatment of myelomonocytic leukemia is suggested. © 1996 by The American Society of Hematology.

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metric assay, the reaction was stopped by adding HCl. The formazan deposits were solubilized by adding DMSO, and the absorption of percentage of cells staining intracellular blue-black formazan deposits was determined by examination of more than 200 cells. NBT reduction was assayed microscopically and colorimetrically and was measured after dissolving the formazan deposits at 560 nm was measured in a spectrophotometer (U-2000; Hitachi, Tokyo, Japan). Lysozyme activity in conditioned medium was determined by a method with lysozymates containing 1% agar, 1/15 mol/L sodium phosphate buffer (pH 5.6), 50 mmol/L NaCl, and heat-killed Micrococcus lysodeikticus (0.5 mg/mL). 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. 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. 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:

$$CI = \frac{(D_1)/(D_{1x}) + (D_2)/(D_{2x})}{(D)/(D_x)}$$

where $D_x$ 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 tocopherol 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. We examined the effect of tretinoin tocopherol on growth and differentiation of HL-60 cells. Tretinoin tocopherol 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 tocopherol for inhibiting cell growth were higher than those of RA (Fig 2B). Both RA and tretinoin tocopherol induced NBT-reducing activity, a typical marker of myelomonocytic differentiation, of the HL-60 cells concentration-dependently (Table 1, Fig 2). In the cells treated with tretinoin tocopherol, 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 tocopherol, 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 tocopherol 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-
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.21 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 $10^{-4}$ 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-

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**Fig 2.** Effects of tretinoin tocoferil (A) and RA (B) on growth and NBT reducing activity of HL-60 cells. Cells (2 x 10⁶/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.
Table 1. Effects of Tretinoin Tocoferil on Differentiation of HL-60 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NBT-Reducing Cells (%)</th>
<th>Pro</th>
<th>Mye</th>
<th>Gr</th>
<th>CD11b-Positive Cells (%)</th>
<th>Viable Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 ± 2</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0 ± 0</td>
<td>96 ± 0</td>
</tr>
<tr>
<td>TT 1.35 × 10⁻⁴ mol/L</td>
<td>77 ± 2</td>
<td>33</td>
<td>41</td>
<td>26</td>
<td>19 ± 1</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>2 × 10⁻⁴ mol/L</td>
<td>69 ± 2</td>
<td>12</td>
<td>36</td>
<td>52</td>
<td>41 ± 11</td>
<td>98 ± 0</td>
</tr>
<tr>
<td>RA 9 × 10⁻⁴ mol/L</td>
<td>92 ± 2</td>
<td>1</td>
<td>25</td>
<td>74</td>
<td>23 ± 2</td>
<td>97 ± 1</td>
</tr>
</tbody>
</table>

Cells (1 to 2 × 10⁶ 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.

活力和表达CD11b表面抗原的细胞由RA诱导（图3，表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 × 10⁻⁴ 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.

图3. α-生育酚对生长抑制（A）和NBT-还原活性（B）的影响。HL-60细胞在RA中与α-生育酚的联合。细胞（2 × 10⁶/mL）用α-生育酚在组合物中与0（Ο），3 × 10⁻⁷（△），9 × 10⁻⁷（■），和3 × 10⁻⁶ mol/L RA（+）共培养4天。值是平均±SD三个独立实验。
<.005) and 5.4 ± 0.2 A560/107 cells (P < .01), respectively. TPA less than 1 nmol/L induced NBT-reducing activity of HL-60 cells. 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 A560/107 cells and tretinoin tocoferil further increased this activity to 6.5 ± 1.0 A560/107 cells (P < .01; Fig 5B). The NBT-reducing activity of HL-60 cells induced by VD3 was increased on combined treatment with tretinoin tocoferil: VD3 (3 × 10−8 mol/L) plus tretinoin tocoferil (3 × 10−7 mol/L) increased the activity to 5.6 ± 1.1 A560/107 cells, which was more than twice that with VD3 alone (2.6 ± 0.3 A560/107 cells) (P < .02) (Fig 5C). Lysozyme activity, another marker of myelomonocytic differentiation, of HL-60 cells was induced concentration-dependently by TPA and VD3, 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 VD3 (data not shown). Thus, tretinoin tocoferil enhanced the differentiation of HL-60 cells induced by DMSO, TPA, or VD3.

Synergistic effects of the combination of VD3 and tretinoin tocoferil in inhibiting proliferation and inducing differentiation of monoblastic U937 cells. VD3 is a promising inducer of differentiation for the treatment of some types of leukemia and cancer. We next examined the effects of VD3 plus tretinoin tocoferil on proliferation and differentiation of human monoblastic U937 cells. VD3 inhibited proliferation of U937 cells concentration-dependently, its IC50 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, VD3 inhibited cell growth more effectively (Fig 6A). As shown in Table 3, 9 × 10−8 mol/L tretinoin tocoferil decreased the IC50 concentration of VD3 to 4.26 × 10−10 mol/L (18.6% of that without tretinoin tocoferil). Figure 6B shows isoboles for the combination of VD3 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 × 10−8 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 VD3, this concentration range effectively induced the activity: VD3 at 3 × 10−9 mol/L did not induce appreciable activity (0.5 ± 0.1 ± 0.6 ± 0.0 A560/107 cells for control cells), but in combination with 4 × 10−7 mol/L tretinoin tocoferil, it induced activity of 4.7 ± 0.5 A560/107 cells (P < .0005; Fig 6C). Thus VD3 and tretinoin tocoferil in combination showed synergistic effects in inhibiting proliferation and inducing differentiation of U937 cells.

Effects of VD3 plus tretinoin tocoferil on proliferation and differentiation of several myelomonocytic leukemia cells. As shown in Fig 7, VD3 induced NBT-reducing activity in human myelomonoblastic ML-1, monocytic THP-1, P39/TSU, and P31/PUJ 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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NBT-Reducing Cells (%)</th>
<th>CD11b-Positive Cells (%)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−RA</td>
<td>+RA</td>
<td>−RA</td>
</tr>
<tr>
<td>None</td>
<td>0 ± 0</td>
<td>42 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>α-Tocopherol 3 × 10−4 mol/L</td>
<td>0 ± 0</td>
<td>58 ± 5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>TT 3 × 10−6 mol/L</td>
<td>2 ± 1</td>
<td>69 ± 1</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

Cells (2 × 106 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.
Fig 5. Effects of the combination of tretinoin tocoferil with DMSO (A), TPA (B), or VD3 (C) on NBT-reducing and lysozyme activities of HL-60 cells. Cells (2 × 10⁵ cells/mL) were treated with DMSO, TPA, or VD3 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 VD3 were 4.2 ± 0.3 versus 4.4 ± 0.3 absorbance/10⁷ cells for cells without the solvents, 3.8 ± 0.4 versus 3.4 ± 0.1 absorbance/10⁷ cells, respectively. The solvents did not effectively increase the lysozyme activities in the cells treated with TPA or VD3 (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 VD3 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 × 10⁻⁴ mol/L VD3 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 VD3 plus tretinoin tocoferil for 6 days (Table 3). The IC₅₀ concentrations of VD3 for the myelomonocytic leukemia cells were decreased by combined treatment with VD3 and tretinoin tocoferil (Fig 7). Lysozyme activity was induced in P39/TSU and P31/FUJ cells by 3 × 10⁻⁴ mol/L VD3 and tretinoin tocoferil at 9 × 10⁻⁸ mol/L slightly increased these activities (data not shown). The IC₅₀ concentrations of VD3 for inhibiting proliferation of these myelomonocytic leukemia cells were decreased by combined treatment with VD3 and tretinoin tocoferil in inhibiting proliferation. Addition of 9 × 10⁻⁸ mol/L tretinoin tocoferil decreased the IC₅₀ concentration of VD3 for inhibiting proliferation to 10⁻¹⁰ mol/L level in all the cells examined. Thus, tretinoin tocoferil and VD₃ 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. Because colchicine inhibited the induction of migration, tretinoin tocoferil was suggested to stimulate chemotaxis of macrophage. 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. Tretinoin tocoferil enhanced the differenti-
Table 3. Effects of Combination Treatment With Tretinoin Tocoferil and VD₃ on Proliferation of Human Myelomonocytic Leukemia Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC₅₀ of TT (mol/L)</th>
<th>IC₅₀ of VD₃ (mol/L)</th>
<th>IC₅₀ of Both (mol/L)</th>
<th>CI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937</td>
<td>1.05 x 10⁻⁸</td>
<td>2.29 x 10⁻⁸</td>
<td>4.28 x 10⁻¹⁰</td>
<td>0.27</td>
</tr>
<tr>
<td>HL-60</td>
<td>6.26 x 10⁻⁹</td>
<td>3.35 x 10⁻⁹</td>
<td>6.32 x 10⁻¹⁰</td>
<td>0.17</td>
</tr>
<tr>
<td>ML-1</td>
<td>6.61 x 10⁻⁹</td>
<td>5.48 x 10⁻¹⁰</td>
<td>2.42 x 10⁻¹⁰</td>
<td>0.58</td>
</tr>
<tr>
<td>THP-1</td>
<td>9.22 x 10⁻⁹</td>
<td>3.38 x 10⁻⁹</td>
<td>7.79 x 10⁻¹⁰</td>
<td>0.33</td>
</tr>
<tr>
<td>P38/TSU</td>
<td>1.17 x 10⁻⁹</td>
<td>2.36 x 10⁻⁹</td>
<td>7.04 x 10⁻¹⁰</td>
<td>0.38</td>
</tr>
<tr>
<td>P31/FUJ</td>
<td>1.44 x 10⁻⁹</td>
<td>1.48 x 10⁻⁹</td>
<td>7.57 x 10⁻¹⁰</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Cells (2 x 10⁴ cells/mL of U937, HL-60, THP-1, P38/TSU, and P31/FUJ cell lines and 4 x 10⁴ cells/mL of ML-1 cell line) were cultured with test compounds for 6 days. IC₅₀ values are concentrations of TT required for 50% inhibition of cell growth or concentrations of VD₃ required for 50% inhibition of cell growth in the absence or presence of TT (9 x 10⁻⁶ mol/L).

* Combination index at IC₅₀. CI values with a fixed concentration of TT (9 x 10⁻⁶ 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.

Effects of a-tocopherol on the differentiation of HL-60 cells induced by RA (Fig 4, Table 2), and the CI value of a combination of 9 x 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 a-tocopherol inhibited differentiation of mouse myeloid leukemia M1 cells induced by dexamethasone. Then, we examined whether a-tocopherol adversely affected differentiation of HL-60 cells. a-Tocopherol did not inhibit proliferation or induce the differentiation of HL-60 cells, but enhanced the differentiation induced by RA (Fig 3, Table 2). a-Tocopherol acid succinate was reported to induce differentiation of HL-60 cells and mouse melanoma cells.

On the other hand, Sokoloski et al. reported that a-tocopherol acid succinate alone did not induce differentiation of HL-60 cells, but it enhanced the differentiation induced by VD₃. Thus, a-tocopherol and its derivative have differentiation-enhancing activity on some cells. Although tretinoin tocoferil is an a-tocopherol ester of RA, it was reported to be stable. 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% of it was unchanged and that RA was not detected. Tretinoin tocoferil was not hydrolyzed by in vitro treatment with esterase. These findings suggest that tretinoin tocoferil affects the growth and differentiation of leukemia cells without catalyzing to a-tocopherol and RA.

Retinoids, including RA, are reported to have toxicity and teratogenicity. The LD₅₀ 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. Oral administration of RA at more than 15 mg/kg/d for 12 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. The long-term treatment with tretinoin tocoferil at 300 mg/kg/d did not induce hypertriglyceremia, which is commonly induced by active retinoids including RA and 13-cis retinoic acid. 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. 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. 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. 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 x 10⁻⁷ mol/L tretinoin tocoferil plus 3 x 10⁻⁹ mol/L VD₃, as shown in Fig 6C, induced NBT reduction to 4.7 ± 0.5 % of normal cells, while the treatment with 4 x 10⁻⁷ mol/L RA plus 3 x 10⁻⁹ mol/L VD₃ induced the reduction to the same level, 4.7 ± 0.3 % of normal cells (data not shown). Tretinoin tocoferil, when combined with VD₃, 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. These findings indicate that tretinoin tocoferil has more therapeutic potency than RA. Besa et al. reported that administration of a-tocopherol ameliorated the toxicity of 13-cis retinoic acid in a clinical trial to myelodysplastic syndrome. Therefore, a-tocopherol esterification of RA may contribute to reducing the toxicity without abolishing the differentiation-enhancing activity.
**DIFFERENTIATION BY TRETINOIN TOCOFERIL**

**Fig 6.** Effects of tretinoin tocoferil in combination with VD₃ on growth and NBT-reducing activity of U937 cells. (A) Growth of U937 cells treated with 0 (○), 3 × 10⁻⁹ (△), or 9 × 10⁻⁹ mol/L tretinoin tocoferil (□). (B) Isoboles for combination of tretinoin tocoferil with VD₃ that are isoeffective (IC₅₀) for the inhibition of proliferation of U937 cells. The dashed line indicates the zero interaction isobole. Cells (2 × 10⁴ cells/mL) were treated with tretinoin tocoferil and VD₃ for 6 days in (A) and (B). (C) NBT reducing activity of U937 cells. Cells (1 × 10⁶ cells/mL) were treated with tretinoin tocoferil in the absence (○) or presence of 3 × 10⁻⁹ mol/L VD₃ (△) for 4 days. Values are the mean (±SD) for three separate experiments.

VD₃ is a promising inducer for differentiation.⁵⁴ Although VD₃ 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 VD₃ to induce differentiation of leukemia cells.⁵¹,⁵² Similar to VD₃, 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 VD₃ on NBT-reducing activity of ML-1 (A), THP-1 (B), P39/TSU (C), and P31/FUJ cells (D). Cells (2 x 10⁶ cells/mL) were treated with VD₃ and 0 (□), 9 x 10⁻⁸ (▲), or 9 x 10⁻⁷ 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 VD₃ 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 10⁻¹⁰ mol/L level and VD₃ at the 10⁻¹⁰ mol/L level synergistically inhibited proliferation of human myelomonocytic leukemia cells, the combined treatment with VD₃ and tretinoin tocoferil may be useful in therapy of myelomonocytic leukemia.

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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

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