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Greater Synergism of Retinoic Acid Receptor (RAR) Agonists with Vitamin D_3 Than That of Retinoid X Receptor (RXR) Agonists with Regard to Growth Inhibition and Differentiation Induction in Monoblastic Leukemia Cells

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ABSTRACT. Retinoids and 1α,25-dihydroxyvitamin D_3 (VD_3) cooperatively induce the differentiation of myeloid leukemia cells. We investigated the role of retinoid receptors (RARs and RXRs) in the combined effects of retinoids and VD_3 on growth inhibition and differentiation induction in human monoblastic leukemia U937 cells by using RAR- or RXR-selective retinoids. An isobologram analysis showed that both combinations were synergistic with regard to inhibiting the proliferation, and RAR agonists exhibited greater synergism with VD_3 than did RXR agonists. RXR agonists alone induced nitroblue tetrazolium (NBT) reduction and expression of CD11b in U937 cells, whereas RAR agonists alone did not. On the other hand, RAR agonists and RXR agonists enhanced the differentiation induced by VD_3, but RXR agonists required higher concentrations. An RAR antagonist inhibited the differentiation induced by RAR agonists plus VD_3, but not that induced by RXR agonists plus VD_3. Thus, RARs and RXRs act differently in their synergism with VD_3. RAR agonists are more potent than RXR agonists with regard to synergism with VD_3, and their combination may be useful in differentiation therapy against myeloid leukemia. BIOCHEM PHARMACOL **57**;5:521–529, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. leukemia; differentiation; RAR; RXR; vitamin D_3

ATRA§, an active natural retinoid, is used successfully in differentiation therapy against APL [1], and can induce the granulocytic differentiation of cells derived from APL and other types of myeloid leukemia [2–4]. ATRA exerts various biologic actions through specific nuclear receptors (RAR-α, RAR-β, and RAR-γ) [5]. APL cells have a genetic rearrangement between the *RAR-α* gene and the nuclear protein *pml* gene, which may contribute to both leukemogenesis and clinical responsiveness to ATRA [6, 7]. Another natural retinoid, 9CRA, binds to RARs and other retinoid receptors (RXR-α, RXR-β, and RXR-γ) [5]. 9CRA has been reported to induce the differentiation of myeloid leukemia cells more effectively than ATRA [8]. Since retinoid receptors are expressed in normal cells throughout the body, the administration of a high dose of retinoid induces several adverse effects, especially in the liver, in the central nervous system, and in embryonic development [9, 10]. Retinoic acids have not been used to treat patients with myeloid leukemia other than APL.

Vitamin D is a potential inducer of differentiation therapy against myeloid leukemia. An active form of vitamin D, VD_3, induces monocytic differentiation of several myeloid leukemia cells and can prolong the survival of mice inoculated with murine myeloid leukemia cells [11–13]. Although the administration of 1α-hydroxyvitamin D_3, which is metabolized to an active form in the liver, has been reported to prevent the progression of myelodysplastic syndrome to overt leukemia, the use of VD_3 is limited, because it produces hypercalcemia [14, 15]. VD_3 binds to VDR, a member of the nuclear receptor family, and its biological actions are mediated mainly by the VDR/RXR heterodimer [16]. The combination of VD_3 with other drugs, including a ligand for the nuclear receptor family, is one approach to overcome its adverse effects.

AMoL is more resistant to intensive chemotherapy than other types of acute myeloid leukemia, and cytotoxic intensive chemotherapy induces several complications, including disseminated intravascular coagulation syndrome, in AMoL patients as well as in APL patients [17]. AMoL is

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§ *Abbreviations*: ATRA, all-*trans* retinoic acid; VD_3, 1α,25-dihydroxyvitamin D_3; RAR, retinoic acid receptor; RXR, retinoid X receptor; 9CRA, 9-*cis* retinoic acid; VDR, vitamin D_3 receptor; AMoL, acute monocytic leukemia; APL, acute promyelocytic leukemia; PPAR, peroxisome-proliferator-activated receptor; and NBT, nitroblue tetrazolium.

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a potential target for differentiation therapy. Although ATRA is less effective in inducing the differentiation of AMoL cells, the combination of ATRA with VD_3 synergistically induces the differentiation of human monoblastic U937 cells [2, 18]. An ATRA derivative, tretinoin tocoferil, also inhibits proliferation and induces the differentiation of U937 cells synergistically with VD_3 [19]. The combination of 9CRA and VD_3 is more potent than that of ATRA and VD_3 in inducing the differentiation of U937 cells [20, 21]. In this study, to clarify the role of RARs and RXRs in the effects of the combination of retinoids and VD_3 on growth inhibition and differentiation induction, we examined the effects of the combination of RAR- or RXR-selective retinoids and VD_3 in the human monoblastic U937 cell line. We also discuss the therapeutic potency of these selective retinoids in combination with VD_3 in differentiation therapy for AMoL.

MATERIALS AND METHODS

Materials

ATRA was purchased from the Sigma Chemical Co., 9CRA from Biomol Research Laboratories, and VD_3 from Wako Pure Chemical Industry. The stock solutions for ATRA and 9CRA were 4×10^{-3} M and that for VD_3 was 1.2×10^{-3} M in ethanol. RAR-selective retinoids (Am80, Am580, Ch55, Re80, and Am555s) were synthesized as previously described [22–24], and their stock solutions were 3×10^{-3} M in ethanol. RXR-selective retinoids (Ro47–5944 and Ro48–2250) and an RAR antagonist (Ro41–5253) were obtained from F. Hoffmann–La Roche. The final concentrations of ethanol in all experiments were below 0.5%, which did not affect cell proliferation or differentiation.

Cell Lines and Cell Culture

Human monoblastic U937 cells were cultured in suspension in RPMI 1640 medium containing 10% fetal bovine serum and 80 μg/mL of gentamicin at 37° in a humidified atmosphere of 5% CO_2 in air.

Cell Growth and NBT-Reducing Activity

Suspensions of cells were cultured with or without the test compounds in multidishes. The cells were counted in a model ZM Coulter Counter (Coulter Electronics). NBT reduction was assayed colorimetrically by a method reported by Takuma *et al.* [25] and modified in our laboratory [19]. Briefly, cells were incubated with 1 mg/mL of NBT (Sigma) and 100 ng/mL of phorbol-12-myristate 13-acetate (Sigma) in RPMI 1640 medium at 37° for 30 min, and the reaction was stopped by adding HCl (final concentration 1 M). Formazan deposits were solubilized in DMSO (Wako), and absorption of the formazan solution at 560 nm per 10^7 cells was measured in a spectrophotometer (U-2000; Hitachi).

Flow Cytometry

Expression of the antigens for myeloid differentiation, CD11b and CD14, on the cell surface was determined by indirect immunofluorescent staining and flow cytometry [26]. Mouse monoclonal antibodies to CD11b (2LPM19c), CD14 (TÜK4), and control mouse IgG1 and IgG2a were obtained from Dako. Cells were treated with the mouse monoclonal antibody in IFA buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 4% fetal bovine serum, and 0.1% NaN_3) plus 2% Block Ace (Snow Brand Milk Products), and stained with an FITC-conjugated $F(ab')_2$ fragment of goat antimouse IgG (Dako) in IFA buffer plus 2% Block Ace. The stained cells were assayed using a flow cytometer (Epics XL; Coulter Electronics). Mean fluorescence intensity was calculated using the Immuno-4 histogram analysis program (Coulter), with mouse immunoglobulin of the same isotype as a negative control. The Immuno-4 program subtracts a control histogram from a test histogram to calculate the mean fluorescence intensity in the test histogram [27].

Cell Cycle Analysis

The cell cycle was analyzed using propidium iodide staining [28]. Briefly, cells were fixed by the addition of cold ethanol, suspended with 250 μg/mL of RNase A in 1.12% sodium citrate at 37° for 30 min, and stained with 50 μg/mL of propidium iodide (Sigma) on ice for more than 30 min. The stained nuclei were analyzed with an Epics XL flow cytometer.

Analysis of the Effects of Combinations of Drugs

The interaction of two compounds was analyzed using isobolograms [29]. Concentration-dependent effects were determined from isoeffective concentrations for each compound and for one compound with fixed concentrations of another.

Transactivation Assays for RAR, RXR, or VDR

CV-1 cells were transfected with 200 ng of receptor plasmid (pCMX-hRAR-α, pCMX-hRXR-α, or pCMX-VDR), 500 ng of reporter plasmid, and 300 ng of pCMX-β-gal using Lipofectin reagent (Gibco BRL). The reporters used were TK-TREpx2-LUC [30], TK-CRBPII-LUC [31], and TK-Sppx3-LUC [32] for RAR-α, RXR-α, and VDR, respectively. The cells were transfected for 18 hr, and after removing the DNA-containing medium, they were incubated with a test compound in medium containing 10% resin-charcoal-stripped fetal bovine serum for 24 hr. Luciferase and β-galactosidase activities were analyzed using a luminescence reader (BLR-201; Aloka) and a spectrophotometer (Hitachi), respectively. All transfection data were normalized using an internal β-galactosidase marker.

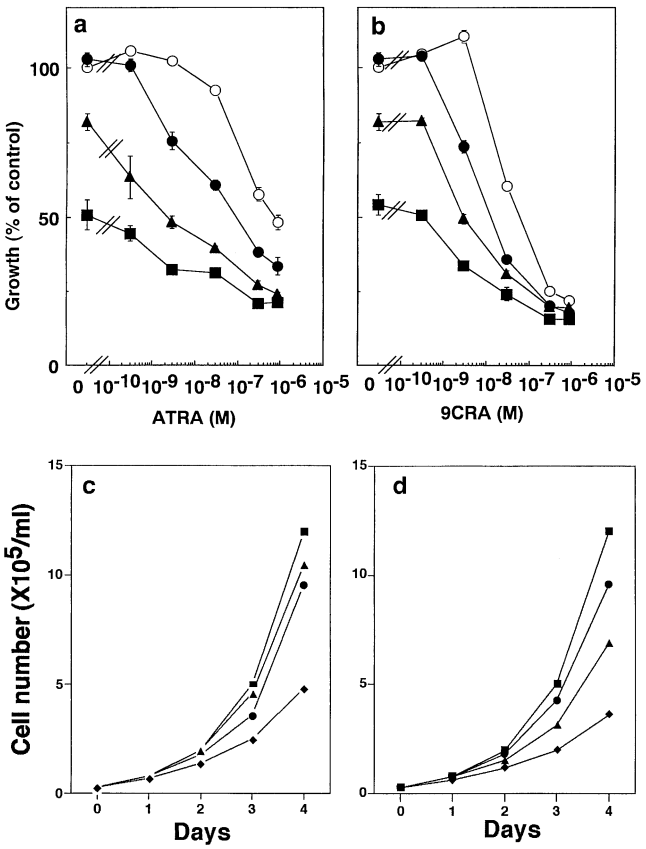

FIG. 1. Effects of ATRA (a and c) and 9CRA (b and d) in combination with VD_3 on growth inhibition in U937 cells. (a and b) Cells (5×10^4 cells/mL) were treated with various concentrations of ATRA or 9CRA in the absence (○) or presence of 3×10^{-10} M (●), 3×10^{-9} M (▲), or 3×10^{-8} M (■) VD_3 for 4 days. The cell number of the control culture at day 4 was 11.6 (±0.4) × 10^5/mL. (c and d) Cells were treated with 3×10^{-9} M VD_3 (●), 3×10^{-8} M ATRA or 9CRA (▲), or VD_3 plus ATRA or 9CRA (♦); (■) untreated cells. Values represent the means ± SD of three separate experiments.

RESULTS

Effects of the Combination of Retinoids and VD_3 on Growth Inhibition of Human Monoblastic U937 Cells

We used human monoblastic U937 cells because this cell line is used as an experimental model for AMoL, and there is greater synergism in the combination of retinoid and VD_3 on growth and differentiation in U937 cells than in other cells, including HL-60 cells [18, 19]. U937 cells were treated with several concentrations of ATRA or 9CRA in combination with VD_3. ATRA at more than 3×10^{-9} M concentration-dependently inhibited the proliferation of U937 cells (Fig. 1, a and c). VD_3 at 3×10^{-10} M and 3×10^{-9} M had little if any effect on the growth inhibition of U937 cells, but enhanced the growth-inhibiting activity of ATRA. 9CRA inhibited proliferation more effectively than ATRA (Fig. 1, b and d). The addition of VD_3 also augmented the growth-inhibiting activity of 9CRA. Next, we compared the effects of combinations on growth inhibition using an isobologram analysis. Isoboles for IC_{50} of growth inhibition induced by the combination of ATRA or 9CRA and VD_3 are plotted in Fig. 2, showing that although both combinations were synergistic, ATRA plus VD_3 was more effective than 9CRA plus VD_3.

To examine the roles of RARs and RXRs in synergism with VD_3 with regard to growth inhibition, RAR-selective retinoids (Am80 and Am580) [22, 23] and RXR-selective retinoids (Ro47–5944 and Ro48–2250) [33] were combined with VD_3. All four combinations exhibited synergistic interaction (Fig. 2). The isobole curve for Am80 plus VD_3 was similar to that for Am580 plus VD_3, and that for Ro47–5944 plus VD_3 was similar to that for Ro48–2250 plus VD_3. RAR agonists were more synergistic with VD_3 with regard to growth inhibition than were RXR agonists. Interestingly, the isobole curve for ATRA was similar to those for RAR agonists, and that for 9CRA was similar to those for RXR agonists.

Effects of Retinoids on the Differentiation of U937 Cells in Combination with VD_3

ATRA slightly induced NBT-reducing activity, which is a typical marker of myeloid differentiation, in U937 cells. However, in the presence of VD_3 it markedly increased this activity (Fig. 3a). When combined with 3×10^{-9} M or 3×10^{-8} M VD_3, ATRA, even at 3×10^{-10} M, induced NBT-reducing activity in U937 cells. 9CRA alone at 3×10^{-6} M to 9×10^{-6} M induced NBT-reducing activity more effectively than ATRA (Fig. 3b). 9CRA was also more effective than ATRA in enhancing the NBT-reducing activity induced by VD_3 (Fig. 3).

Next, we examined the effects of selective retinoids plus VD_3 in inducing the differentiation of U937 cells. RAR agonists (Am80, Am580, Ch55, Re80, and Am555s) induced only marginal NBT-reducing activity in U937 cells, whereas RXR agonists (Ro47–5944 and Ro48–2250) concentration-dependently induced NBT reduction (Fig. 4).

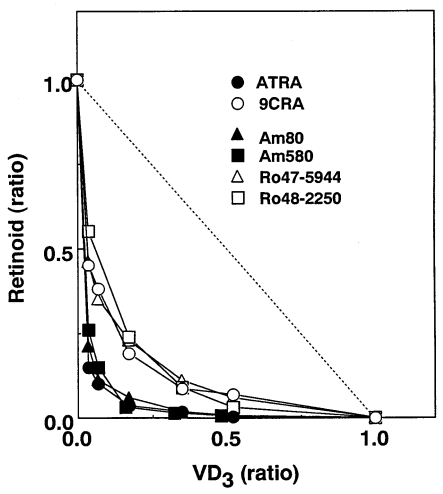

FIG. 2. Isobologram for retinoids and VD_3 at the IC_{50} for growth inhibition in U937 cells. Cells (2×10^4 cells/mL) were cultured with test compounds for 4 days. Values are the means of triplicate data. The dashed line indicates additive interaction.

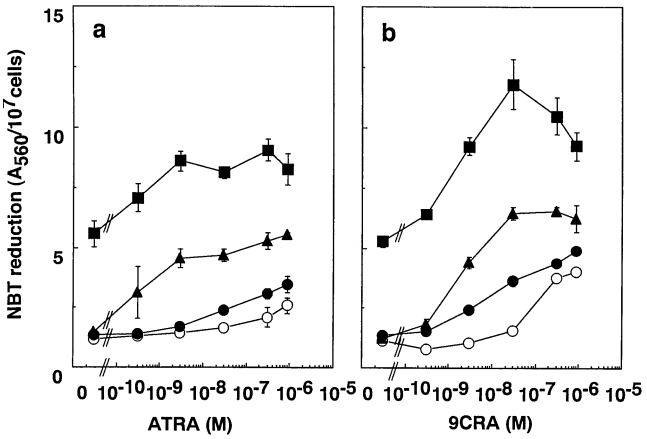

FIG. 3. Effects of ATRA (a) and 9CRA (b) in combination with VD_3 on NBT-reducing activity in U937 cells. Cells (5×10^4 cells/mL) were treated with various concentrations of ATRA or 9CRA in the absence (○) or presence of 3×10^{-10} M (●), 3×10^{-9} M (▲), or 3×10^{-8} M (■) VD_3 for 4 days. Values represent the means ± SD of three separate experiments.

Ro48–2250 also induced expression of CD11b in U937 cells, but Am80 did not (Fig. 5c). However, even at 3×10^{-10} M, RAR agonists effectively enhanced the NBT-reducing activity induced by VD_3 (Fig. 5a). In combination with 3×10^{-9} M VD_3, RAR agonists at low concentrations (3×10^{-10} M or 3×10^{-9} M) induced NBT reduction to almost 5 A_{560}. On the other hand, RXR agonists at up to 3×10^{-8} M did not enhance NBT-reducing activity in U937 cells in the presence of VD_3 (Fig. 5b). However, at 3×10^{-7} M and 3×10^{-6} M, they increased NBT reduction more effectively than RAR agonists at the same concentrations. Thus, in terms of molar concentrations, RAR agonists, except for Am555s, were at least 1000 times more active than RXR agonists in enhancing NBT reduction induced by VD_3 in U937 cells. By itself, Am80, an RAR agonist, did not induce the expression of CD11b antigen in U937 cells, but it did augment the expression induced by VD_3 (Fig. 5c). Am80 at 3×10^{-6} M plus VD_3 at 3×10^{-9} M increased CD11b expression to 5.42 units, which corresponds to that induced by 1.7×10^{-7} M VD_3 alone (data not shown). Ro48–2250, an RXR agonist, at 3×10^{-8} M did not increase CD11b expression or enhance the expression induced by VD_3. However, at 3×10^{-6} M, it increased CD11b expression and enhanced the expression induced by 3×10^{-9} M VD_3 to 6.33 units. The patterns in which RAR and RXR agonists induce CD11b expression in the presence or absence of VD_3 were similar to those for NBT reduction (Figs. 4 and 5). Neither RAR nor RXR agonists induced CD14 expression in U937 cells or augmented the expression induced by VD_3, while VD_3 effectively increased this expression (data not shown). Am80 and Ro48–2250 alone did not affect morphological changes, but they also enhanced the morphological changes in U937 cells induced by VD_3 (data not shown).

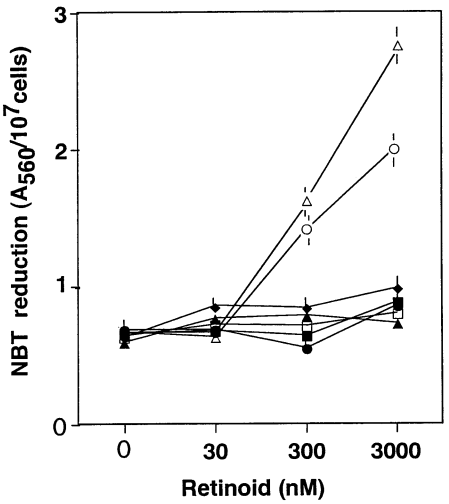

FIG. 4. Effects of various retinoids on induction of NBT-reducing activity in U937 cells. Cells were treated with various concentrations of Am80 (■), Am580 (●), Ch55 (▲), Re80 (♦), Am555s (□), Ro47–5944 (○), or Ro48–2250 (△) for 4 days. Values represent the means ± SD of three separate experiments.

The effects of selective retinoids in combination with VD_3 on the cell cycle of U937 cells were examined (Table 1). Ro48–2250 plus VD_3 effectively increased the percentage of cells in G_1 phase, while Am80 plus VD_3 did not. These results suggest that the pattern of differentiation induced by RAR agonists is different from that induced by RXR agonists in the presence or absence of VD_3.

Effect of an RAR Antagonist on the Differentiation of U937 Cells Induced by Selective Retinoids Plus VD_3

We examined the effects of Ro41–5253, an RAR antagonist [34], on the NBT-reducing activity of U937 cells induced by RAR- or RXR-selective retinoids plus VD_3. Ro41–5253 concentration-dependently inhibited the NBT reduction induced by Am80 plus VD_3 or Am580 plus VD_3, but did not affect that induced by Ro47–5944 plus VD_3 or Ro48–2250 plus VD_3 (Fig. 6). Ro41–5253 alone did not induce activity or inhibit that induced by VD_3 alone (data not shown). Thus, the effect of Ro41–5253 on the differentiation induced by RAR agonists plus VD_3 is different from its effect on the differentiation induced by RXR agonists plus VD_3.

The effects of Ro41–5253 on the differentiation induced by ATRA plus VD_3 or 9CRA plus VD_3 were also examined. The NBT reduction induced by ATRA plus VD_3 was inhibited by Ro41–5253 (Fig. 7). Ro41–5253 also inhibited the NBT reduction induced by 9CRA plus VD_3, but less effectively than that induced by ATRA plus VD_3 (Fig. 7).

Induction of Transactivation via RAR, RXR, and VDR by Selective Retinoids or VD_3

CV-1 cells were transiently transfected with an RAR-α receptor plasmid and a luciferase reporter plasmid, and the effects of Ro41–5253 on luciferase activity induced by

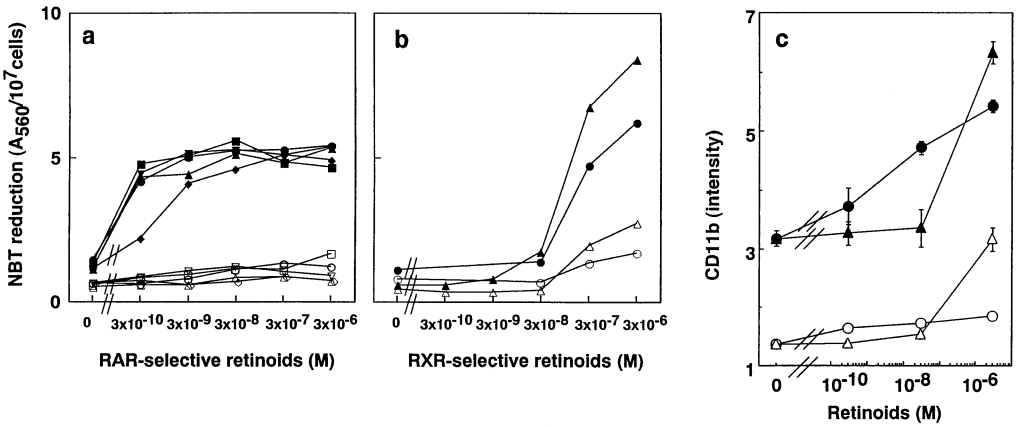

FIG. 5. Effects of RAR-selective and RXR-selective retinoids in combination with VD_3 on differentiation in U937 cells. NBT-reducing activity induced by (a) RAR-selective retinoids [Am80 (○), Am580 (△), Ch55 (□), Re80 (▽), Am555s (♦)] or (b) RXR-selective retinoids [Ro47–5944 (○) or Ro48–2250 (△)] in the absence (open symbols) or presence of 3×10^{-9} M VD_3 (closed symbols). (c) CD11b expression induced by Am80 (○) or Ro48–2250 (△) in the absence (open symbols) or presence of 3×10^{-9} M VD_3 (closed symbols). Cells (5×10^4 cells/mL) were treated with test compounds for 4 days. Values represent the means ± SD of three separate experiments.

selective retinoids were examined. Am80 concentration-dependently induced the transactivation via RAR-α (Fig. 8a). Ro-48–2250 only slightly activated RAR-α, and Ro41–5253 did not (Fig. 8b). The luciferase activity induced by Am80 was inhibited by Ro41–5253. Ro48–2250 induced transactivation via RXR-α (Fig. 8c). The transactivation induced by Ro48–2250 was not affected by Ro41–5253 (Fig. 8d). Next, CV-1 cells were transfected with a VDR expression plasmid and a VDR-responsive luciferase reporter to examine the effects of selective retinoids on transactivation induced by VD_3. VD_3 concentration-dependently induced the luciferase activity (Fig. 8e). Ro48–2250 inhibited the VD_3-induced transactivation, whereas Am80 did not affect the activity.

DISCUSSION

We previously reported the effects of tretinoin tocoferil, which is a less toxic derivative of retinoid, plus VD_3 on growth inhibition and differentiation induction of human myeloid leukemia cells, including U937 cells [19]. Other authors have also reported the combination of retinoids and VD_3 for inducing the differentiation of U937 cells [18, 35]. 9CRA has been reported to enhance the differentiation induced by VD_3 more effectively than ATRA [20, 21]. In this study, we examined the effects of RAR- or RXR-selective retinoids in combination with VD_3 on the proliferation and differentiation of U937 cells and found that different combinations had different effects (Table 2). Isobolograms for growth inhibition could be divided into two patterns: the RAR pattern and the RXR pattern.

TABLE 1. Cell cycle distribution in U937 cells treated with RAR- or RXR- selective retinoids in combination with VD_3

Treatment	Growth (% of control)	Cell cycle (%)		
		G_1	G_2/M	S
None	100	41 ± 3	13 ± 0	46 ± 3
Am80	53	37 ± 8	16 ± 4	47 ± 3
Ro48-2250	25	49 ± 6	13 ± 4	38 ± 2
VD_3	82	38 ± 4	18 ± 2	44 ± 2
VD_3 + Am80	27	41 ± 5	22 ± 3	37 ± 2
VD_3 + Ro48-2250	20	73 ± 1	10 ± 0	17 ± 2

Cells (5×10^4 cells/mL) were treated with 3×10^{-6} M RAR-selective Am80 or RXR-selective Ro48-2250 in the presence or absence of 3×10^{-9} M VD_3 for 4 days. Values represent the means ± SD of three separate experiments.

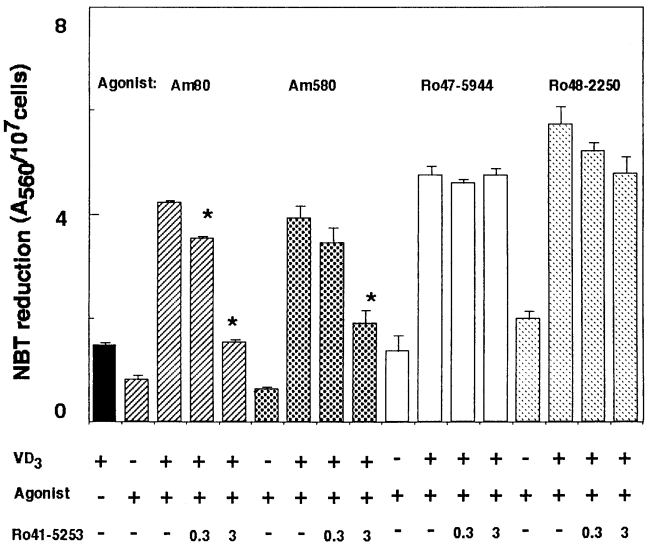

FIG. 6. Effect of an RAR antagonist on NBT-reducing activity induced by RAR-selective or RXR-selective retinoids in combination with VD_3 in U937 cells. The RAR antagonist Ro41–5253, at 0.3 or 3 μM, was combined with 3×10^{-9} M Am80, 3×10^{-9} M Am580, 3×10^{-7} M Ro47–5944, or 3×10^{-7} M Ro48–2250 in the presence or absence of 3×10^{-9} M VD_3. Cells (5×10^4 cells/mL) were treated with the test compounds for 4 days. Values represent the means ± SD of four separate experiments. Key: (*) $P < 0.05$ compared with the combination of retinoid and VD_3 by an unpaired two-tailed Student's *t*-test.

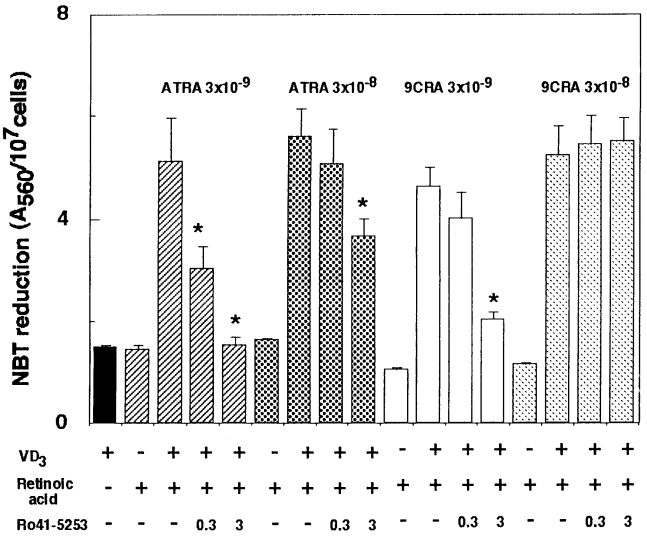

FIG. 7. Effect of an RAR antagonist on NBT-reducing activity induced by ATRA and 9CRA in combination with VD_3. The RAR antagonist Ro41–5253, at 0.3 or 3 μM, was combined with 3×10^{-9} M or 3×10^{-8} M ATRA or 9CRA plus 3×10^{-9} M VD_3. Cells (5×10^4 cells/mL) were treated with test compounds for 4 days. Values represent the means ± SD of four separate experiments. Key: (*) $P < 0.05$ compared with the combination of retinoic acid plus VD_3 by an unpaired two-tailed Student's *t*-test.

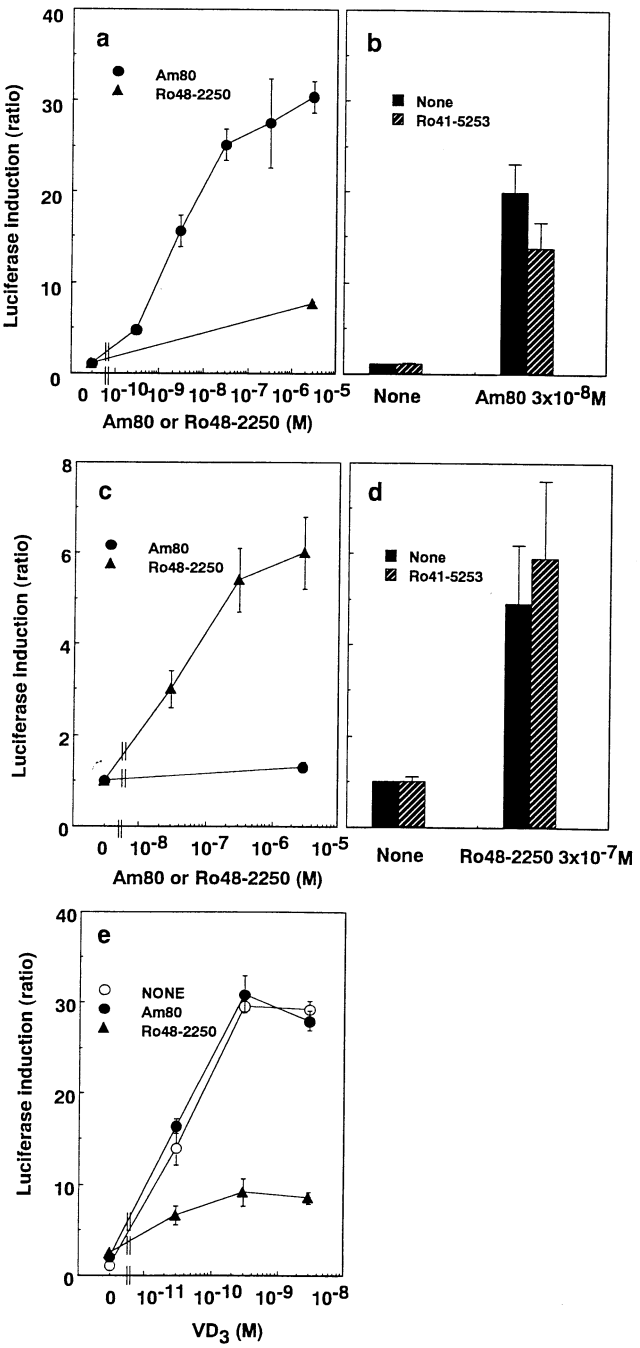

FIG. 8. Induction of transactivation of RAR, RXR, and VDR by retinoids and VD_3. (a and b) Cells were transfected with RAR-α and TK-TREpx2-LUC plasmids. (c and d) Cells were transfected with RXR-α and TK-CRBPII-LUC plasmids. Transfectants were treated with 3×10^{-7} M Ro41–5253 in the presence of 3×10^{-8} M Am80 (b) or 3×10^{-7} M Ro48–2250 (d). (e) Cells were transfected with VDR and TK-Sppx3-LUC plasmids, and then treated with 3×10^{-6} M Am80 or Ro48–2250. Luciferase activities were adjusted for efficiency of transfection by cotransfected β-galactosidase activities. Values represent the means ± SD of three separate experiments.

Although both retinoids showed synergism with VD_3, RAR-selective retinoids showed greater synergism with VD_3 than RXR-selective retinoids. Since ATRA is an agonist for RARs, it shows the RAR pattern. Interestingly, although 9CRA binds to both RARs and RXRs, it shows the RXR pattern. 9-*cis* Retinoic acid α-tocopherol ester, which is a 9-*cis* isomer of tretinoin tocoferil that binds to RARs and only weakly to RXRs, demonstrated the RXR pattern (data not shown). Thus, the interaction of a retinoid with RXRs, regardless of binding to RARs, may determine its isobologram pattern. VDR acts by forming a RXR/VDR heterodimer, but the addition of RXR ligand interferes with the formation of the heterodimer and leads to the RXR/RXR homodimer [36]. This competition may contribute to weakening the synergism between RXR agonist and VD_3 with regard to growth inhibition. Thus, RAR agonists are more potent than RXR agonists with regard to synergistic growth inhibition with VD_3.

The RAR agonists used in this study are effective at inducing the differentiation of human promyelocytic leukemia HL-60 cells [22, 23]. RAR-selective retinoids alone were less effective in inducing differentiation markers in U937 cells, but even at low concentrations enhanced the differentiation induced by VD_3. Although RXR-selective retinoids alone at low concentrations were not effective, at high concentrations they induced the differentiation of U937 cells and enhanced the differentiation induced by VD_3 more effectively than RAR-selective retinoids. The RXR-selective compounds at more than 10^{-7} M significantly induced differentiation of U937 cells, whereas the RAR-selective compounds did not at the same concentrations. The RAR antagonist Ro41–5253 inhibited the differentiation induced by RAR agonists plus VD_3 but not that induced by RXR agonists plus VD_3. Ro41–5253 inhibited the differentiation induced by ATRA plus VD_3

TABLE 2. Summary of the effects of the combination of retinoids and VD_3 on growth inhibition and differentiation induction in U937 cells

Receptor selectivity	Retinoids	Growth-inhibiting pattern in isobologram	Effect of Ro41-5253* on differentiation
RAR	ATRA, Am80, Am580, Ch55, Re80, Am555s, tretinoin tocoferil	Strong synergism	Inhibited
RXR	Ro47-5944, Ro48-2250	Moderate synergism	Not changed
RAR and RXR	9CRA	Moderate synergism	Inhibited

*RAR antagonist [30].

and 9CRA plus VD_3, but its inhibitory activity was less with 9CRA plus VD_3. This indicates that the enhancing activity of 9CRA in the differentiation induced by VD_3 is mediated by both RARs and RXRs. Based on the findings in combination with Ro41–5253, the enhancing effects of retinoids on differentiation induced by VD_3 are mediated separately by RARs and RXRs. Combination with VD_3 and RXR ligand effectively induced G_1 arrest of the cells, whereas combination with RAR ligand at the same concentration did not induce G_1 arrest. These results indicate that the effect of an RXR ligand is different from that of an RAR ligand with respect to induction of differentiation and growth inhibition in U937 cells, although we cannot eliminate the possibility that an RXR ligand may partly act as a weak RAR agonist.

Retinoids and VD_3 interact with nuclear receptors. RAR ligand activates RXR/RAR; RXR ligand alone does not activate RXR/RAR, but does enhance the activation of the heterodimer induced by suboptimal concentrations of RAR ligand [37]. VD_3 interacts with RXR/VDR, but RXR ligand dissociates RXR from the heterodimer to form the RXR/RXR homodimer, thus preventing VD_3-induced activation of VDR [36]. Since the RAR ligand and VD_3 do not directly compete on RXR/RAR or RXR/VDR, the synergism between RAR-selective retinoids and VD_3 may not be due to interaction on their receptors. We suggest that the RAR ligand induces the expression of a set of differentiation-related genes with RAR-responsive elements, while VD_3 induces the expression of another set of differentiation-related genes with VDR-responsive elements. VDR-inducible signals may potentiate the RAR-inducible signals responsible for differentiation, which alone are insufficient to induce differentiation in monoblastic cells. On the other hand, RXR agonists alone could induce differentiation of U937 cells, and the differentiation-enhancing effect of RXR agonists in the presence of suboptimal concentrations of VD3 required the same concentrations (relatively higher than those of RAR agonists). Although the RXR ligand interferes with the function of VD_3 in the RXR/VDR heterodimer (Fig. 8), it exhibits synergistic effects with VD_3 with regard to growth inhibition and differentiation induction in U937 cells. The synergism induced by RXR agonists requires relatively higher concentrations. This suggests that RXR agonists activate some RXR/nuclear receptor heterodimer complexes (e.g. PPAR/RXR or RXR/LXR) as well as the RXR/RXR homodimer [16]. RXR ligand may induce the expression of a set of genes associated with differentiation, growth inhibition, or G_1 arrest via the RXR homodimer or heterodimer, but not RXR/VDR. Some of these differentiation-related genes may be regulated separately by several nuclear receptors. A recent report indicates that a combination with PPAR-γ ligand and RXR-specific retinoid promotes myelomonocytic differentiation of leukemia cells [38]. Treatment with either PPAR-γ ligand or RXR ligand alone has a lesser effect. Thus, the synergism of retinoids and VD_3 may be mediated by a combination of several nuclear receptors, such as RXR/RAR, RXR/RXR, RXR/VDR, and RXR/other nuclear receptor.

ATRA is used successfully to treat APL. Although its clinical use is limited to APL, it can induce the differentiation of cells of myeloid leukemias other than APL. Since retinoid receptors are expressed in normal cells throughout the body, the administration of high doses of retinoid induces several adverse effects, which preclude its use against non-APL myeloid leukemia. However, the pattern of expression of retinoid receptors varies among cells. For example, myeloid leukemia cells have RAR-α and RAR-β, but not RAR-γ, and retinoid-related skin toxicity is mediated by RAR-γ. Selective retinoids for RAR-α and RAR-β, such as Am80, induce less skin toxicity [39]. Thus, the use of a selective retinoid is one approach to diminish retinoid-related toxicity. We previously reported that tretinoin tocoferil, which is an α-tocopherol ester of ATRA that has less retinoid-related toxicity, effectively enhances the differentiation in myelomonocytic leukemia cells induced by VD_3 [19]. The enhancing effect of tretinoin tocoferil on the differentiation induced by VD_3 is mediated by RARs, and the isobologram for the combination of tretinoin tocoferil and VD_3 on growth inhibition shows the RAR pattern (data not shown). Thus, the combination of retinoids with other drugs, such as VD_3, can further reduce their toxicity. In this study, RAR-selective agonists synergistically inhibited proliferation and induced the differentiation of U937 cells in combination with VD_3. Such combinations can decrease the required concentrations of RAR-selective retinoid and VD_3 and may reduce the toxicity of each drug. For example, the isobologram shows that the combination of Am80 and VD_3 can reduce the effective concentrations of each drug to almost one-tenth of the respective values alone (Fig. 2). Am80, even at 3×10^{-10} M, effectively enhanced the NBT-reducing activity induced by VD_3 in U937 cells (Fig. 5). Recently, an RXR-selective retinoid,

LGD1069, was reported to not induce retinoid-related toxicity in a clinical trial, suggesting that it may be clinically useful for treating cancer [40]. Although RXR agonists and VD_3 are synergistic with regard to growth inhibition and differentiation induction in U937 cells, RXR agonists are less synergistic than RAR agonists with regard to growth inhibition and require higher concentrations to enhance differentiation markers induced by VD_3. The combination of 9CRA, which is a *pan* agonist for retinoid receptors, with VD_3 effectively induced differentiation in U937 cells, but its broad receptor selectivity may induce several adverse effects in many organs. In addition, the interaction with both RARs and RXRs can weaken the synergism with VD_3 with regard to growth inhibition. Thus, the combination of selective retinoids for RARs, especially RAR-α and RAR-β, such as Am80, with VD_3 may be a promising candidate for differentiation therapy against AMoL. In conclusion, both RAR-selective retinoids and RXR-selective retinoids show synergism with VD_3 with regard to inhibiting proliferation and inducing differentiation in U937 cells, and RAR agonists are more potent than RXR agonists in this synergism with VD_3.

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