# A Retinoic Acid Receptor β/γ-Selective Prodrug (tazarotene) Plus a Retinoid X Receptor Ligand Induces Extracellular Signal-Regulated Kinase Activation, Retinoblastoma Hypophosphorylation, $G_0$ Arrest, and Cell Differentiation

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## ABSTRACT

Retinoic acid receptor (RAR)β is perceived to function as a tumor suppressor gene in various contexts where its absence is associated with tumorigenicity and its presence causes cell cycle arrest. Tazarotene is a prodrug selective for RARβ/γ, thereby motivating interest in determining whether tazarotene might activate putative tumor suppressor activity. Using HL-60 human myeloblastic leukemia cells, a cell line that undergoes $G_0$ cell cycle arrest and myeloid differentiation in response to retinoic acid (RA), tazarotene failed to cause extracellular signal-regulated kinase (ERK) activation, a requirement for retinoic acid (RA)-induced $G_0$ arrest and differentiation; retinoblastoma (RB) hypophosphorylation, another characteristic of RA-induced $G_0$ arrest and cell differentiation; $G_0$ arrest; or differentiation into mature myeloid cells. However, when used in combination with a retinoid X receptor (RXR)-selective ligand, tazarotene caused ERK activation, RB tu- mor suppressor protein hypophosphorylation, $G_0$ arrest, and myeloid differentiation. The kinetics of $G_0$ arrest and differentiation was similar to that of RA. Dose-response studies showed that diminishing tazarotene progressively diminished both induced cell differentiation and $G_0$ arrest, where the doses for cellular effects were consistent with the transcriptional transactivation data. For either tazarotene or an RARα-selective ligand, diminishing the coadministered RXR-selective ligand diminished both induced differentiation and $G_0$ arrest. Tazarotene could propel either early or late portions of the period leading to differentiation and $G_0$ arrest and was interchangeable with an RARα-selective ligand. Tazarotene used with RXR-selective ligand may thus be a useful antineoplastic agent in differentiation induction therapy as exemplified by the prototypical RA treatment of acute promyelocytic leukemia.

There is accumulating evidence that the retinoic acid receptor (RAR)β may have tumor suppressor activity. RARβ is a member of the RAR family, which with the retinoid X receptor (RXR) family, are ligand-activated transcription factors activated by retinoids. Its expression is reduced or lost in a number of tumors (Xu, 2001; Zhang et al., 2001; Zou et al., 2001; Li et al., 2002). Furthermore, in tumor-derived cell lines resistant to retinoic acid-induced growth arrest, increasing or restoring RARβ expression by ectopic expression

confers response to retinoic acid (Si et al., 1996; Wu et al., 1998; Faria et al., 1999; Wan et al., 1999; Weber et al., 1999). The growth inhibitory action of RARβ may in part reflect its ability to inhibit the transcriptional activity of AP-1, the fos and Jun heterodimer whose activity is typically up-regulated with cell proliferation or tumor progression (Lin et al., 2000). These considerations motivate interest in the potential antiproliferative effects of RARβ agonists.

The mechanism of action of retinoic acid, which with its retinoid cellular metabolites causes activation of RARs and RXRs, has been studied in a variety of tumor-derived cell lines. One of the longest studied and perhaps most fully characterized molecularly is the HL-60 human myeloblastic leukemia cell line (Collins et al., 1977; Yen, 1990), which was

**ABBREVIATIONS:** RAR, retinoic acid receptor; RXR, retinoid X receptor; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PBS-T, phosphate-buffered saline-Tween 20; RA, retinoic acid; NBT, nitro blue tetrazolium; Ro40-6055, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-6-naphthalenylcarboxamido)benzoic acid; Ro41-5253, 6-[1-(4-carboxyphenyl)propen-2-yl]-3,4-dihydro-4,4-dimethyl-7-heptyloxy-2H-benzothiopyrene-2,2-dioxide; AGN194301, 4-[[(8-bromo-2,2-dimethyl-4-p-tolyl-2H-chromene-6-carbonyl)-amino]-2-fluoro-benzoic acid.

isolated from a patient with what was retrospectively reevaluated as a myeloblastic (FAB M1) leukemia. RAR$\alpha$ and RAR$\beta$, but not RAR$\gamma$, and RXR$\alpha$ and RXR$\beta$, but not RXR$\gamma$, have been observed in HL-60 cells (De The et al., 1989; Nervi et al., 1989; Hashimoto et al., 1990; Yu et al., 1991; Kizaki et al., 1993) where RAR$\alpha$ protein is expressed at higher levels than RAR$\beta$ protein (Gaub et al., 1989; Hashimoto et al., 1989). HL-60 cells proliferate avidly in culture and undergo $G_0$ cell cycle arrest and either myeloid or monocytic differentiation, depending on the agent they are treated with. Retinoic acid induces myeloid differentiation, and 1,25-dihydroxy vitamin D3 induces monocytic differentiation. The process of induced $G_0$ arrest and differentiation segregates into two segments, an early one that primes the cells to differentiate without lineage specificity and a late one wherein the myeloid or monocytic lineage is determined (Yen et al., 1987; Yen and Forbes, 1990). In the HL-60 subline studied, the onset of $G_0$ arrest and differentiation typically is apparent after 48 h of exposure to RA, a period corresponding approximately to the duration of two cell cycles. The duration of the early period is approximately the duration of one cell cycle and leads to a precommitment state where cells are primed to differentiate without regard to lineage specificity, which is determined during the subsequent late period, the duration of which is also approximately one cell cycle time. Activation of RAR$\alpha$ plus RXRs by receptor-selective ligands results in $G_0$ arrest and myeloid differentiation with kinetics comparable with that elicited by retinoic acid, whereas activation of either RAR$\alpha$ or RXR separately is essentially ineffective (Brooks et al., 1996). Retinoic acid and its presumed cellular metabolites thus need to activate RAR$\alpha$ and RXR to cause $G_0$ arrest and myeloid differentiation of HL-60 cells. Likewise, retinoic acid also causes RAR$\alpha$ plus RXR-dependent prolonged activation of the ERK2 MAPK, which is necessary to elicit $G_0$ arrest and differentiation (Yen et al., 1998, 1999). Inhibiting MAPK signaling abrogated retinoic acid-induced growth arrest and differentiation, whereas augmenting it enhanced retinoic acid-induced arrest and differentiation. One of the hallmarks of retinoic acid-induced MAPK-dependent cellular response, namely, $G_0$ arrest and myeloid differentiation, is the conversion of hyperphosphorylated RB tumor suppressor protein to the hypophosphorylated form (Yen et al., 1994, 1996, 1997a,b; Brooks et al., 1996; Yen and Soong, 1996). It is not known whether RAR$\beta$ can propel any of these processes, although it might be suspected given its potential tumor suppressor activity.

A significant amount of data indicates that RAR$\alpha$ activation is seminal to retinoic acid-induced differentiation. For example, ectopic expression of RAR$\alpha$ in HL-60 cells resistant to retinoic acid induced differentiation restored retinoic acid-inducible differentiation (Mehta et al., 1997). In wild-type HL-60 cells, RXR/RXR homodimer activation had little effect on differentiation or growth arrest, but activation of RAR/RXR heterodimers with RXR/RXR homodimers resulted in growth inhibition and differentiation (Kizaki et al., 1996). Also suggesting the importance of RAR$\alpha$ specifically, ectopic expression in murine marrow progenitor cells of RAR$\alpha$, its dominant negative and its fusion proteins, PML-RAR$\alpha$, PLZF-RAR$\alpha$, and NPM-RAR$\alpha$, found in myeloid leukemias, showed that altering the RAR$\alpha$ pathway caused growth perturbations, but overexpression of RAR$\beta$, RAR$\gamma$, or RXR$\alpha$ did not (Du et al., 1999). In WEHI murine myeloid leukemia

cells, inhibiting retinoic acid-induced loss of RAR$\alpha$ protein with either granulocyte colony-stimulating factor or LiCl enhanced resulting differentiation (Finch et al., 2000). Although a significant amount of data thus indicates the importance of RAR$\alpha$ activation to eliciting retinoic acid-induced growth arrest and differentiation, the case for RAR$\beta$ is not as well studied.

Tazarotene is ethyl 6-[2-(4, 4-dimethylthiochroman-6-yl)-ethynyl] nicotinate, a drug used for the treatment of psoriasis. It is metabolized by a ubiquitous esterase to its active form, tazarotenic acid, a RAR$\beta$/$\gamma$-selective retinoid. In keratinocytes, tazarotene was found to induce the expression of TIG3, a putative class II tumor suppressor gene 52% identical to H-rev 107 (DiSepio et al., 1998). It presents an opportunity to study consequences of RAR$\beta$-activated pathways in HL-60 cells. The present results show that it has potential antineoplastic activity if used in differentiation induction therapy.

## Materials and Methods

**Cells and Culture Conditions.** HL-60 human myeloblastic leukemia cells (Collins et al., 1977) were continuously cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (Intergen, Purchase, NY) as described previously (Brooks et al., 1996; Yen et al., 1998, 1999). Stock cells were maintained in 10-ml cultures that were initiated at a density of $0.2 \times 10^6$ cells/ml for 2 days, twice a week, and then $0.1 \times 10^6$ cells/ml for 3 days, once a week, to sustain constant exponential growth.

Experimental 30-ml cultures were initiated at a cell density of $0.2 \times 10^6$ cells/ml with retinoic acid (Sigma-Aldrich, St. Louis, MO), 1,25-dihydroxy vitamin D3 (Solvay Duphar B.V., Weesp, The Netherlands) or retinoids as indicated. Retinoic acid or 1,25-dihydroxy vitamin D3 was added from a $10^{-3}$ M stock in ethanol stored at $-20°C$ protected from light. Retinoids were likewise added, as described previously (Brooks et al., 1996), from 5 mM stocks that were solubilized in ethanol and stored at $-80°C$ protected from light. AGN90169, also called tazarotene, an RAR$\beta$/$\gamma$-selective ligand; and AGN194301, an RAR$\alpha$-selective antagonist, were a generous gift of Allergan, Inc. (Irvine, CA). Ro40-6055, an RAR$\alpha$-selective ligand, also known as AM580; Ro25-7386, an RXR-selective ligand that can activate transcription through RXR$\alpha$, but not RXR$\gamma$; and Ro41-5253, an RAR$\alpha$ antagonist (Brooks et al., 1996), were generous gifts of F. Hoffmann-La Roche (Nutley, NJ). In experiments treating cells with retinoic acid or 1,25-dihydroxy vitamin D3, the final concentration in culture was $1.0 \times 10^{-6}$ M retinoic acid and $0.5 \times 10^{-6}$ M 1,25-dihydroxy vitamin D3. All retinoids were used at a final concentration of $1.0 \times 10^{-6}$ M unless otherwise noted as follows. In dose-response experiments treating cells with different doses of tazarotene, the final concentrations of tazarotene were $1.0 \times 10^{-6}$, $1.0 \times 10^{-7}$, $1.0 \times 10^{-8}$, and $1.0 \times 10^{-9}$ M. In dose-response experiments treating cells with different doses of RAR$\alpha$ agonist, the final concentrations of RAR$\alpha$ agonist were $1.0 \times 10^{-6}$, $1.0 \times 10^{-7}$, $1.0 \times 10^{-8}$, and $1.0 \times 10^{-9}$ M. In experiments treating cells with an RAR$\alpha$ agonist, RXR agonist, and an RAR$\alpha$ antagonist, AGN194301, the final concentrations in culture of the antagonist were $1.0 \times 10^{-9}$ or $1.0 \times 10^{-8}$ M. In experiments with cells treated with either an RAR$\alpha$ agonist plus an RXR agonist or tazarotene plus an RXR agonist in the absence or presence of an RAR$\alpha$ antagonist, Ro41-5253, the final concentration of RAR$\alpha$ agonist was $1.0 \times 10^{-8}$ M. In experiments where cells were treated with tazarotene plus an RXR agonist for 24 h and then an RAR$\alpha$ agonist plus an RXR agonist thereafter, the entire culture was harvested by centrifugation at 24 h, resuspended, and washed twice in approximately 20 ml of warmed (37°C) serum-supplemented medium, incubated for 10 min, and then resuspended in the original volume of fresh medium containing the second treat-

ment as described previously (Brooks et al., 1996). Parallel cultures were treated with RAR$\alpha$ agonist in lieu of tazarotene as a control. In experiments where tazarotene was used as the second rather than the first treatment, the same protocol was used. In RXR dose-response experiments treating cells with either tazarotene or an RAR$\alpha$ agonist plus different concentrations of RXR agonist, the concentration of tazarotene and RAR$\alpha$ agonist was $1.0 \times 10^{-6}$ M, and the final concentrations of RXR agonist were $1.0 \times 10^{-6}$, $1.0 \times 10^{-7}$, and $1.0 \times 10^{-8}$ M. At the indicated times after initiation of culture/treatment,

cells were harvested to determine cell density, differentiation, cell cycle distribution, or Western analysis. All experiments shown are typical of two or more repeats.

**Assays of Growth and Differentiation.** Assays of cell growth by measuring cell density and distribution in the cell cycle, and assays of cell differentiation detected by inducible oxidative metabolism were performed as described previously (Brooks et al., 1996; Yen et al., 1998; Yen et al., 1999). In brief, cell density in experimental cultures was measured by repeated counts with a hemacytometer. Viability was assessed by exclusion of 0.2% trypan blue dye and was routinely at least 95% in all cultures. The distribution of cells in the cell cycle was determined by flow cytometry using propidium iodide-stained nuclei. Cells ($0.5 \times 10^6$) were harvested at each indicated time and resuspended in 0.5 ml of hypotonic propidium iodide solution (0.05 mg/ml propidium iodide, 1 mg/liter sodium citrate, and 0.1% Triton X-100) and stored refrigerated and protected from light until analyzed. Flow cytometric analysis was done with a multiparameter dual laser fluorescence activated cell sorter (EPICS 753; Beckman Coulter, Fullerton, CA) using 200 mW of 488-nm excitation from a tunable argon ion laser. Functional differentiation to a mature myelo-monocytic phenotype capable of inducible oxidative me-

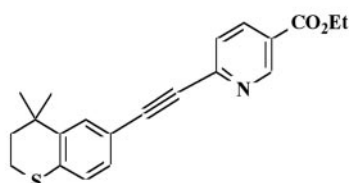**Chemical Name and Structure:**
Tazarotene



**Fig. 1.** Chemical structure of tazarotene.



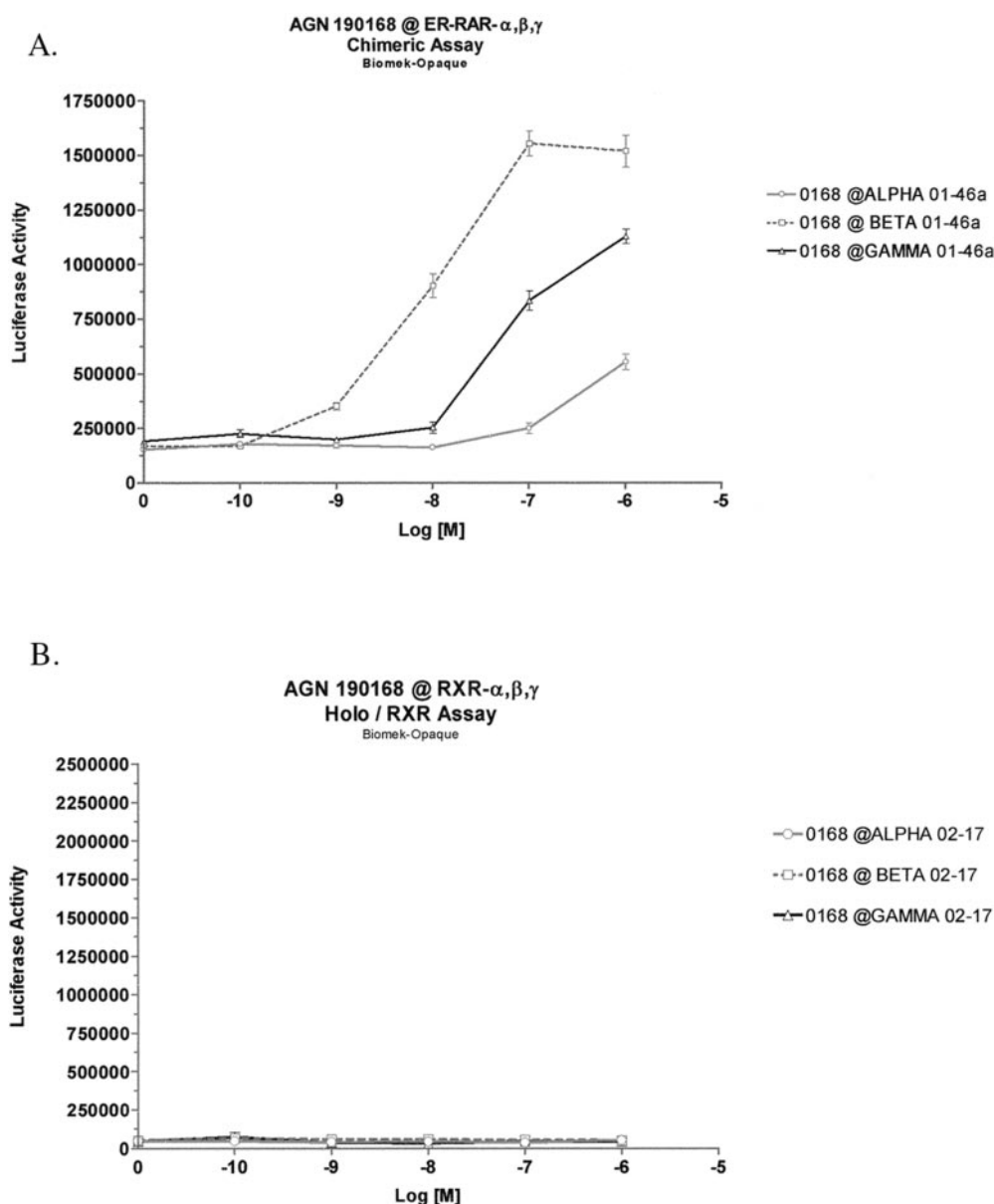**Fig. 2.** Transcriptional activation through RAR$\alpha$, RAR$\beta$, RAR$\gamma$ and RXR$\alpha$, RXR$\beta$, RXR$\gamma$ by tazarotene. A, transcriptional activation of luciferase reporter activity by tazarotene through RAR$\alpha$ (circle), RAR$\beta$ (square), and RAR$\gamma$ (triangle) in a chimeric receptor assay. B, transcriptional activation of luciferase reporter activity through RXR$\alpha$ (circle), RXR$\beta$ (square), and RXR$\gamma$ (triangle) in a holoreceptor assay. Horizontal axis, logarithm of tazarotene concentration. Vertical axis, luminometer-measured luciferase activity.

tabolism was assayed by phorbol 12-myristate 13-acetate (Sigma-Aldrich)-inducible oxidative metabolism resulting in intracellular reduction of nitroblue tetrazolium to formazan by superoxide. Cells $(0.2 \times 10^6)$ were harvested at the indicated times and resuspended in 0.2 ml of 2 mg/ml nitroblue tetrazolium in PBS containing 200 ng/ml phorbol 12-myristate 13-acetate in dimethyl sulfoxide. The cell suspension was incubated for 20 min in a 37°C water bath and then scored using a hemacytometer for the percentage expressing intracellular purple formazan precipitated by superoxide. Over 200 cells were counted per sample, and variation in replicates is routinely within 10%.

**Western Analysis of RB and Activated MAPK.** Western blotting was done using whole cell lysates from cells as described previously (Brooks et al., 1996; Yen et al., 1998; Hong et al., 2001). At indicated times, $10^6$ cells were harvested and fixed in 1 ml of 90% methanol at $-20$°C. The cells were stored at $-20$°C until analysis by SDS-PAGE. Cells were solubilized in 50 $\mu$l of loading buffer (6% SDS, 4 M urea, 4 mM EDTA, 125 mM Tris, pH 6.9, 0.25% bromphenol blue, and 35 $\mu$l/ml $\beta$-mercaptoethanol) by boiling in a water bath for 5 min. SDS-PAGE was done using a 4% stacking gel and a 10% resolving gel with 37.5:1 acrylamide/bis. Samples were electrophoresed for 1200 V-hours., typically 75 V for 16 h. Then, $1 \times 10^6$ cells were loaded per lane. Proteins were electrotransferred (Trans Blot Cell; Bio-Rad, Hercules, CA) from the gel to a nitrocellulose membrane. Transfer was done at 0.8 amps for 1 h. The resulting membrane was blocked by overnight immersion at 4 C in 5% powdered milk and 0.05% Tween 20 in PBS (PBS-T). The membranes were stained with Ponceau S dye to check uniformity of lane loading and electrotransfer as described previously (Hong et al., 2001). The membranes were probed with antibodies detecting the phosphorylated and unphosphorylated forms of RB and the activated ERK2 and 1 bearing a TEY motif with T(183) and Y(185) phosphorylation. The antibody to detect RB (RB Gene Product (mAb1) Monoclonal Antibody; Zymed Laboratories, South San Francisco, CA) was used at 0.4 mg/ml PBS-T with an overnight incubation at 40°C. The antibody used to detect activated ERK1 and 2 (#V6671 rabbit polyclonal antibody; Promega, Madison, WI) was used at 0.025 $\mu$g/ml in 0.1% bovine serum albumin in PBS-T with a 2-h incubation at room temperature. Detection was performed using a horseradish peroxidase-conjugated secondary anti-mouse or -rabbit antibody and enhanced chemiluminescence (ECL kit; Amersham Biosciences Inc., Piscataway, NJ) following the manufacturer's instructions.

**Transactivation Assays.** Transcriptional activation assays of RAR$\alpha,\beta,\gamma$ and RXR$\alpha,\beta,\gamma$ were performed as described previously using transient transfection to coexpress the specific receptor subtype and a promoter-reporter with luciferase read out (Nagpal et al.,

1995). In brief, transcriptional activation through RARs by tazarotene was characterized by a chimeric receptor assay in which CV-1 cells were transiently transfected with a promoter-reporter construct, ERE-tk-Luc, bearing a luciferase reporter under control of an estrogen receptor binding site that responds only to retinoids, plus the expression vector for an ER-RAR fusion protein in which the DNA binding domain of the RAR, either $\alpha$, $\beta$, or $\gamma$, was replaced by an estrogen receptor DNA binding domain. Treating cells with tazarotene at the indicated concentrations resulted in the reported luciferase activity. Transcriptional activation through RXRs were measured in the holoreceptor activity assay performed by transiently transfecting with an RXR-responsive reporter plasmid, CRBP II-TK-LUC, which contains DR1 elements from the human CRBP II promoter plus an RXR expression vector. A $\beta$-galactosidase expression vector was used as an internal control for transfection efficiency. The chimeric receptor assay was used in the case of RARs and the holoreceptor assay in the case of RXRs because CV-1 cells have an endogenous RAR-RXR heterodimer activity, which use of the chimeric receptor assay circumvents; but there is no detectable RXR-RXR homodimer activity, which allows use of the holoreceptor assay.

## Results

Motivated by the putative tumor suppressor activity of RAR$\beta$, the experiments to be described characterize how RAR$\beta$ activation through tazarotene affects the growth and differentiation of a human myeloblastic leukemia cell line. The first experiments show the selective RAR$\beta$-transactivating capability of tazarotene, whose chemical structure is shown in Fig. 1. RA-induced ERK phosphorylation and RB hypophosphorylation are prerequisite for RA-induced cell differentiation and $G_0$ cell cycle arrest, and the next experiments show that tazarotene by itself fails to cause enhanced ERK phosphorylation or hypophosphorylation of RB like RA but that it does when used with an RXR-selective ligand. The next experiments then show that tazarotene by itself fails to cause differentiation or arrest, but it does when used with an RXR-selective ligand. Dose-response studies then show that
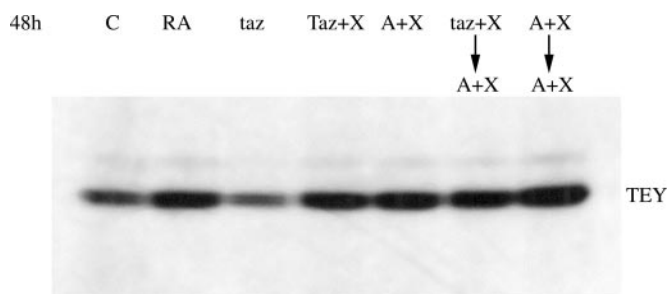
**Fig. 3.** Western blot of activated ERK. HL-60 cells were treated for 48 h with nothing (C, untreated control), RA, tazarotene (taz), tazarotene plus RXR-selective agonist (taz+X), RAR$\alpha$-selective agonist plus RXR-selective agonist (A+X), or tazarotene plus RXR-selective agonist for 24 h and then RAR$\alpha$-selective agonist plus RXR-selective agonist from 24 to 48 h; and as a control where tazarotene is replaced by RAR$\alpha$-selective agonist, RAR$\alpha$-selective agonist plus RXR-selective agonist (A+X) for 24 h and then washed and recultured in RAR$\alpha$-selective agonist plus RXR selective agonist again. Concentration of all agents was 1 $\mu$M. The experiment was repeated twice with consistent results; typical results are shown. This is true for all the figures.
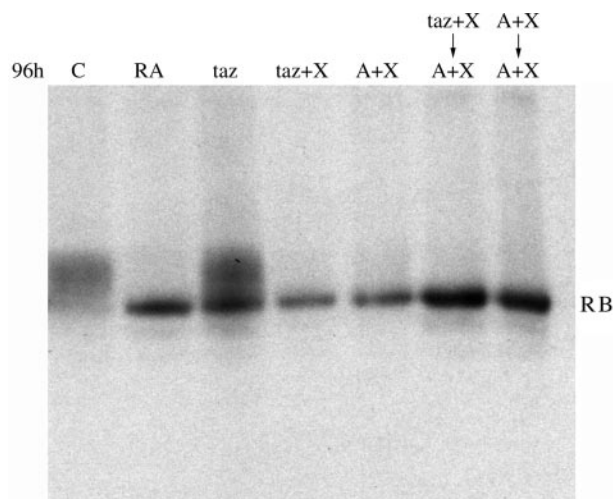
**Fig. 4.** Western blot of RB tumor suppressor protein. HL-60 cells treated for 96 h with nothing (C, untreated control), RA, tazarotene (taz), tazarotene plus RXR-selective agonist (taz+X), RAR$\alpha$-selective agonist plus RXR-selective agonist (A+X), or with tazarotene plus RXR-selective agonist for 24 h and then RAR$\alpha$-selective agonist plus RXR-selective agonist from 24 to 48 h; and as a control where tazarotene is replaced by RAR$\alpha$-selective agonist, RAR$\alpha$-selective agonist plus RXR-selective agonist for 24 h and then washed and recultured in RAR$\alpha$-selective agonist plus RXR-selective agonist again. The experiment was repeated twice with the consistent typical results shown. This is true for all figures.

the induced differentiation and $G_0$ arrest are both sensitive to diminishing doses of tazarotene, where the doses for cellular effects are consistent with the transactivating doses. Likewise dosing down the RXR-selective ligand used with tazarotene also attenuated induced differentiation and $G_0$ arrest. Sequential drug treatment experiments then show that tazarotene and the RARα-selective ligand are interchangeable during the early or late phases of the period before induced differentiation and $G_0$ arrest. The last experiments show that tazarotene used with an RXR-selective ligand or with both RARα plus RXR-selective ligands is unable to cause an RA-resistant cell to differentiate and $G_0$ arrest. In sum, tazarotene (AGN190168) is an RARβ and to a much lesser extent RARγ- or RARα-activating retinoid that synergizes with an RXR agonist to cause ERK activation, RB hypophosphorylation, differentiation, and $G_0$ arrest of HL-60 human myeloblastic leukemia cells.

Tazarotene is an RARβ-selective transactivator. Figure 2 shows the transactivation of RARα, RARβ, RARγ and RXRα, RXRβ, RXRγ. As described previously (Nagpal et al., 1995), CV-1 cells were cotransfected with a promoter reporter construct and the respective receptor and then treated with the indicated concentrations of tazarotene. Luciferase reporter activity was then measured. The luciferase activity induced by tazarotene shows that it causes transcriptional activation through RARβ, is approximately 1.5 logs less effective at activating through RARγ, almost 3 logs less effective at activating through RARα, and largely ineffective at activating through RXRs. In this assay, $10^{-8}$ M resulted in approximately half-maximal transcriptional activation through RARβ, but essentially no detectable transcriptional activation through RARα or RARγ.

Used to treat HL-60 cells, tazarotene by itself was ineffective at causing ERK activation or RB hypophosphorylation, but synergized with an RXR agonist (Ro25-7386, an RXR-selective ligand with RXRα transactivating activity) to activate ERK and to shift RB from the hyperphosphorylated to the hypophosphorylated state. RXRα,β,γ agonists by themselves have previously been shown to be ineffective at causing either ERK activation (Hong et al., 2001) or conversion of RB from the hyperphosphorylated to the hypophosphorylated state (Brooks et al., 1996). Figure 3 shows the Western blot of phospho-ERK using an antibody specific for T(183)EY(185) phosphorylated activated ERK, an assay that correlates with both phosphorylation-induced gel electrophoretic mobility retardation and in vitro kinase activity as reported previously (Yen et al., 1998). HL-60 cells were treated with RA, tazarotene, tazarotene plus an RXR agonist, or an RARα-selective agonist (Ro40-6055) plus an
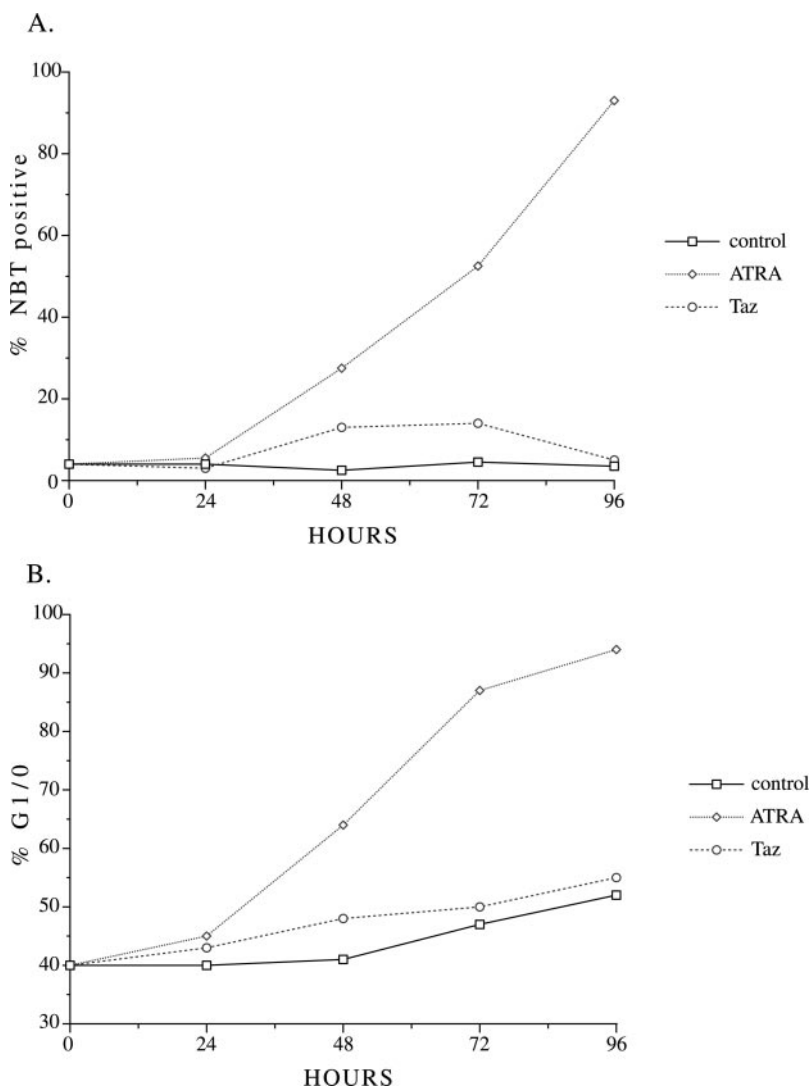


**Fig. 5.** Cell differentiation and cell cycle arrest after tazarotene treatment. Percentage of cells that were differentiated (A) or with $G_{1/0}$ DNA (B) in untreated control (circle), 1 μM RA-treated (square), or 1 μM tazarotene-treated (triangle) HL-60 cells as a function of duration of treatment. Vertical axis, percent. Differentiation was measured as the percentage of cells capable of inducible oxidative metabolism (i.e., superoxide production detected by reducing NBT, a functional differentiation marker characteristic of mature myeloid cells). Relative number of $G_{1/0}$ DNA cells was measured by flow cytometry. Horizontal axis, duration of treatment (hours). For differentiation, over 200 cells are counted per sample, and variation in replicates is routinely within 10%. For cell cycle, $10^4$ cells are analyzed per sample, and variation in replicates is less than 10%. A typical case is shown.

RXR agonist for 48 h. The cells were harvested and whole cell lysate was resolved by PAGE and phospho-ERK detected by Western blotting. The blot shows that although tazarotene by itself failed to augment ERK activation, when used in combination with an RXR agonist, it augmented ERK activation to a comparable degree as RA or the combination of an RARα agonist plus an RXR agonist. The synergism of tazarotene plus an RXR agonist was corroborated by induced RB protein hypophosphorylation. Figure 4 shows the Western blot of RB protein expression. HL-60 cells treated with the above-mentioned agents for 96 h were analyzed by Western blotting for RB protein expression. The gel mobility of hyperphosphorylated RB is retarded relative to hypophosphorylated RB, which is apparent as a faster migrating discrete band. Although untreated

HL-60 cells showed little hypophosphorylated RB protein, RA caused loss of most hyperphosphorylated RB and accrual of hypophosphorylated RB, as reported previously (Yen et al., 1994, 1996, 1997a,b; Brooks et al., 1996; Yen and Soong, 1996). Tazarotene by itself was largely ineffective at causing loss of hyperphosphorylated RB, but with some accrual of hypophosphorylated RB. In contrast tazarotene administered with an RXR agonist caused loss of hyperphosphorylated RB and accrual or hypophosphorylated RB similar to RA or treatment with an RARα-selective ligand plus an RXR ligand. Tazarotene was thus by itself ineffective at inducing molecular responses that characterize cellular differentiation and $G_0$ arrest induced by RA. However, when used with an RXR-selective ligand, it caused ERK activation and RB hypophosphorylation.
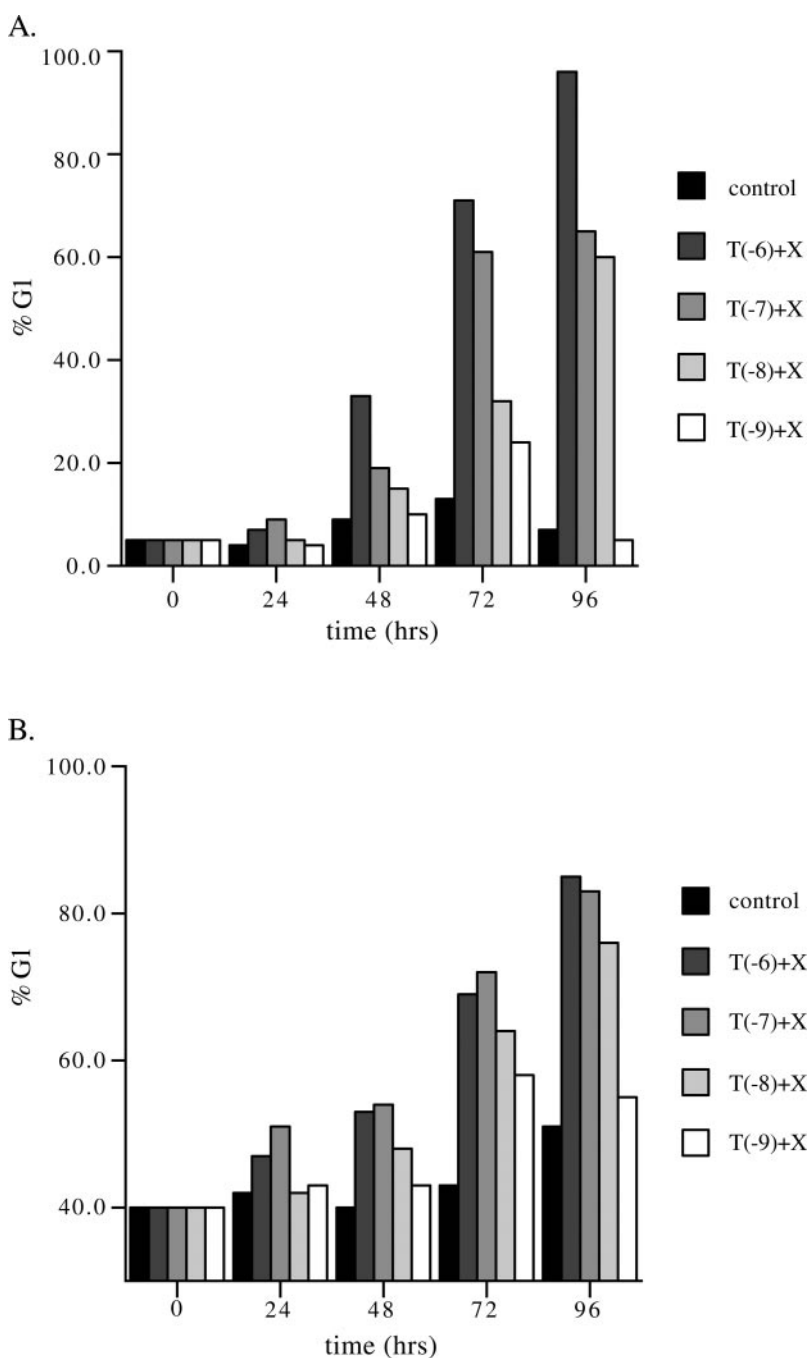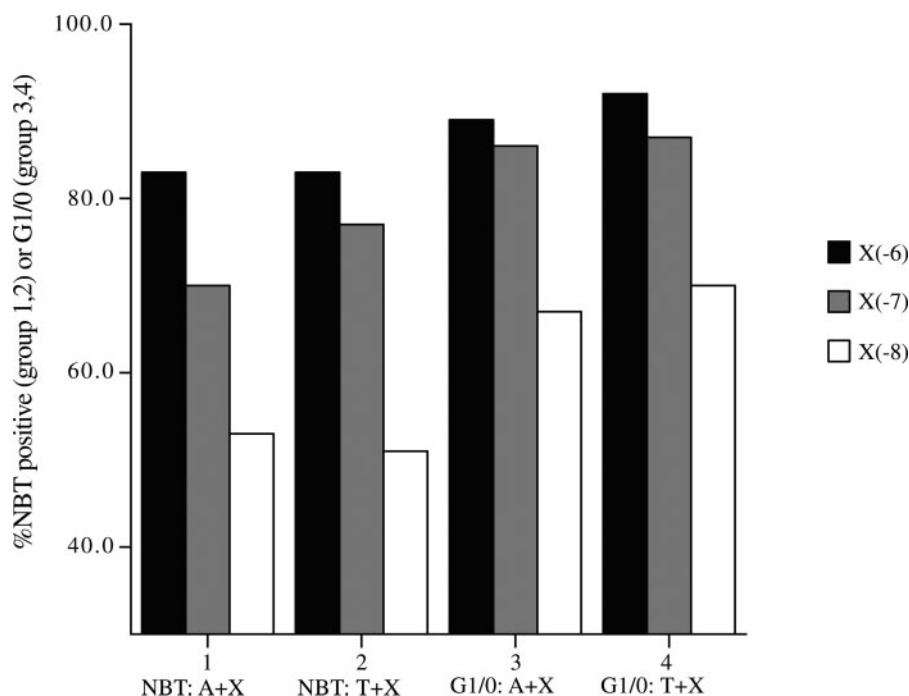


**Fig. 6.** Cell differentiation and cell cycle arrest after tazarotene plus RXR-selective agonist treatment. Tazarotene dose-response relationships. Percentage of cells that were differentiated (A) or with $G_{1/0}$ DNA (B) in untreated control (black), 1 μM tazarotene plus RXR-selective agonist (dark gray), 0.1 μM tazarotene plus RXR-selective agonist (medium gray), 0.01 μM tazarotene plus RXR agonist (light gray), or 0.001 μM tazarotene plus RXR agonist treated HL-60 cells (white) as a function of duration of treatment. Vertical axis, percent. Differentiation and relative number of $G_{1/0}$ DNA cells are as described in Fig. 5 legend. Horizontal axis, duration of treatment (hours).

Tazarotene by itself is not effective at causing cell differentiation and $G_0$ cell cycle arrest, but it synergizes with an RXR agonist to induce differentiation and $G_0$ arrest. It has been previously shown that an RXR agonist by itself is ineffective at causing differentiation or $G_0$ arrest (Brooks et al., 1996). HL-60 cells were cultured with tazarotene, and samples were harvested at 24-h intervals to determine the fraction of cells capable of inducible oxidative metabolism, a functional differentiation marker that characterizes mature myeloid cells, and also the percentage of cells with $G_{1/0}$ DNA, where $G_0$ cell cycle arrest is betrayed by an enrichment thereof. For comparison, cells were also treated with retinoic acid, which induces myeloid differentiation and $G_{1/0}$ arrest. The control cells were untreated. Figure 5 shows the percentage of cells that were capable of inducible oxidative metabolism, detected as the percentage of cells capable of intracellular reduction of NBT to formazan, and the percentage of cells with $G_{1/0}$ DNA, detected by flow cytometry of propidium iodide-stained nuclear DNA. RA caused onset of differentiation and $G_{1/0}$ arrest by 48 h that progressed until the population was largely differentiated and arrested by 96 h. By comparison, tazarotene caused only a small increment in differentiated cells by about 48 h and likewise in the case of $G_0$ arrest. In contrast, tazarotene used with an RXR-selective agonist caused cell differentiation and $G_{1/0}$ cell cycle arrest comparable with that of retinoic acid. HL-60 cells were treated with tazarotene plus an RXR-selective agonist and cell differentiation and $G_{1/0}$ arrest were assayed at 24-h intervals as before. To ascertain dose-response effects, the concentration of tazarotene was decreased by logs from $10^{-6}$ to $10^{-9}$ M. Figure 6 shows the percentage of differentiated cells and cells with $G_{1/0}$ DNA. Used with an RXR-selective agonist, tazarotene, at $10^{-6}$ M, caused onset of cell differentiation by 48 h. The percentage of differentiated cells progressively increased thereafter with almost all cells differentiated by 96 h. Likewise tazarotene plus an RXR-selective agonist induced $G_{1/0}$ enrichment, characterizing cell cycle

arrest. The kinetics of induced cell differentiation and $G_0$ arrest was comparable with that of RA.

Reducing the amount of tazarotene used diminished the amount of both cell differentiation and cell cycle arrest as measured by the percentage of cells capable of inducible oxidative metabolism and by the percentage of cells in $G_{1/0}$. At 96 h, after a 2 log reduction in dose to $10^{-8}$ M, tazarotene still caused at least half the maximum cell differentiation and $G_0$ arrest; but after a further reduction in dose to $10^{-9}$ M, tazarotene had little effect on differentiation or arrest (Fig. 6). It is interesting that the $10^{-8}$ and $10^{-9}$ M tazarotene concentrations are those where transcriptional activation (Fig. 1) through RARβ was diminished by approximately half and then lost, respectively, compared with the maximum elicited by $10^{-6}$ M. The $10^{-8}$ M concentration was also the concentration where there was still significant transcriptional activation through RARβ, but none detectable through RARα or RARγ, in the promoter-reporter assays described above (Fig. 2). Tazarotene used with an RXR-selective agonist could thus propel cell differentiation and $G_0$ arrest with kinetics similar to that of RA. The cellular dose response coincidentally roughly paralleled the dose response for transcriptional activation through RARβ.

Diminishing the RXR agonist dose diminishes induced cell differentiation and $G_0$ arrest for both coadministered RARα agonist or tazarotene. HL-60 cells were treated with either tazarotene or an RARα-selective agonist in the presence of varying concentrations of the RXR agonist, and cells were harvested for analysis of differentiation and cell cycle arrest after 96 h. Figure 7 shows the percentage of differentiated cells as the RXR agonist concentration decreases by logs from $10^{-6}$ to $10^{-8}$ M. The percentage of cells with $G_{1/0}$ DNA is also shown under these conditions. Diminishing RXR agonist caused diminished differentiation as well as $G_{1/0}$ arrest for both tazarotene and the RARα-selective agonist. The RXR agonist dose-dependent responses are roughly similar for both tazarotene and the RARα agonist, consistent with sim-
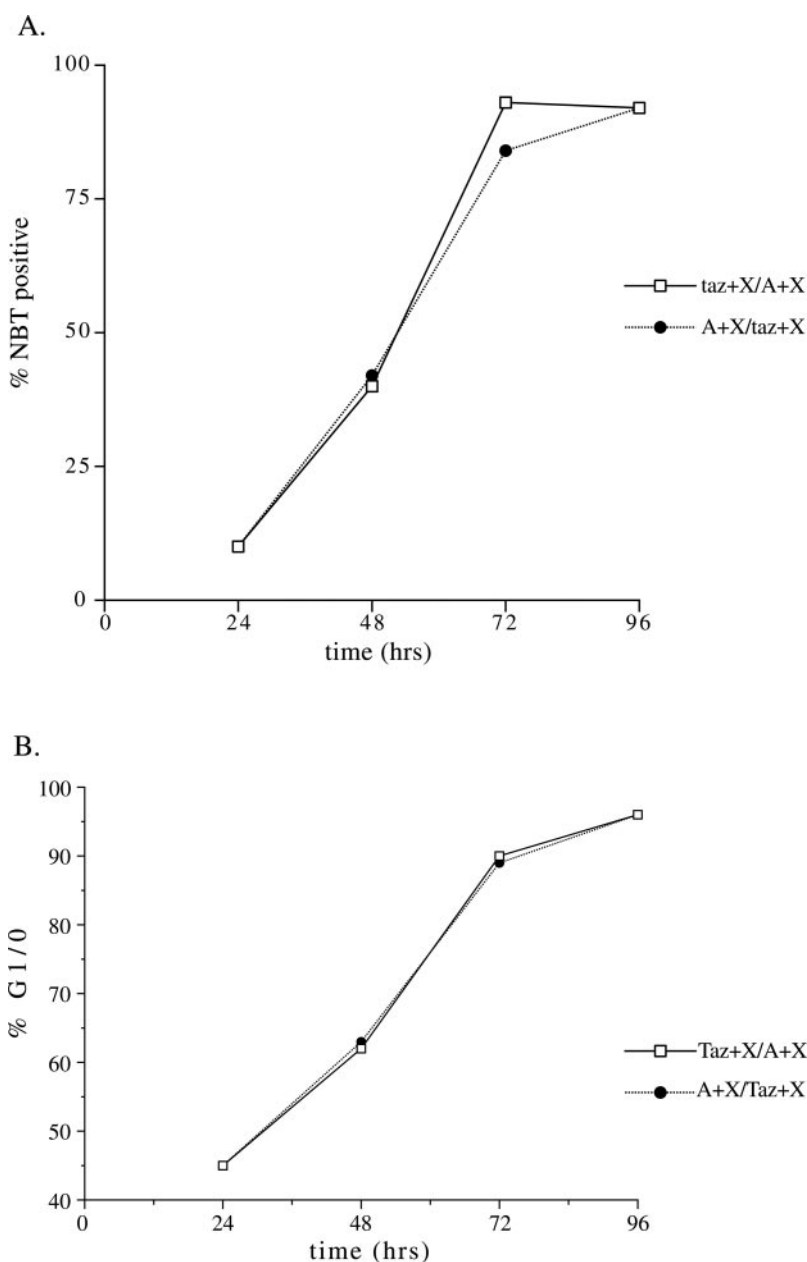


**Fig. 7.** Cell differentiation and cell cycle arrest after treating with different doses of RXR-selective agonist plus either RARα-selective agonist or tazarotene. RXR-selective agonist concentrations were $10^{-6}$ M (black), $10^{-7}$ M (gray), or $10^{-8}$ M (white). Differentiation (NBT) and cells with $G_{1/0}$ DNA ($G_{1/0}$) in HL-60 cells treated for 96 h with RARα-selective agonist plus RXR-selective agonist (A+X), tazarotene plus RXR-selective agonist (T+X). Vertical axis, percent. Differentiation (NBT) and relative number of $G_{1/0}$ DNA cells ($G_{1/0}$) are as described in Fig. 5 legend.

ilar dependencies on RXR activation for both RARα and RARβ to induce differentiation and arrest.

In the HL-60 subline studied, it is known that the onset of $G_0$ arrest and differentiation typically is apparent after 48 h of exposure to RA or 1,25-dihydroxy vitamin D3, a period that segregates into two distinct segments, early (precommitment) and late events. Tazarotene effects early events leading to the precommitment primed state, as well as late events leading from precommitment to onset of terminal differentiation and $G_0$ arrest, which are equivalent to those effected by an RARα-selective agonist. HL-60 cells were cultured either with tazarotene plus an RXR agonist for 24 h to effect precommitment priming and then with an RARα agonist plus an RXR agonist thereafter or with an RARα agonist plus an RXR agonist first and then tazarotene plus an RXR agonist. If the tazarotene and RARα agonist cause equivalent cellular effects during the early and the late segments, leading to differentiation and $G_0$

arrest, then the kinetics of induced differentiation and $G_0$ arrest should be similar for the two cases. Figure 8 shows the typical percentage of differentiated cells and cells with $G_{1/0}$ DNA during treatment in the two cases. The kinetics of induced differentiation and $G_0$ arrest were indistinguishable for the two cases. In contrast to 24, 48, and 96 h, the deviation from coincidence at 72 h is experimental variation and not a feature of repeats. Tazarotene and the RARα agonist thus effected equivalent cellular consequences during the early and late period, leading to differentiation and $G_0$ arrest. Corroborating this, ERK activation, which is known to propel differentiation and arrest, was apparent after sequential treatment at 48 h (Fig. 3). Likewise, RB protein was converted from the hyperphosphorylated to the hypophosphorylated state (Fig. 4). The data are consistent with the suggestion that RARα and RARβ activation can function as equivalent alternatives for causing differentiation and $G_0$ arrest.



**Fig. 8.** Cell differentiation and cell cycle arrest after treating with either tazarotene plus RXR-selective agonist for 24 h and then RARα-selective agonist plus RXR-selective agonist thereafter (square, Taz+X/A+X), or, reversing the order of treatment, RARα-selective agonist plus RXR-selective agonist for 24 h and then tazarotene plus RXR-selective agonist thereafter (circle, A+X/Taz+X). Vertical axis, percent. Differentiation (A) and relative number of $G_{1/0}$ DNA cells (B) are as described in Fig. 5 legend. Horizontal axis, duration of treatment (hours).

Tazarotene plus an RXR agonist was unable to overcome resistance to induced differentiation and $G_0$ arrest in an HL-60 cell subline made resistant to RA by prolonged culture in progressively increasing amounts of RA. The cells, HL-60R−/D+, were thus resistant to RA, but differentiation and $G_0$ arrest caused by 1,25-dihydroxy vitamin D3 was not impaired. Although it has been suggested that RA resistance frequently arises because of a mutational hot spot causing truncation of the RARα receptor, the truncation, which causes conversion of a fok1 site to a premature stop codon, was not detected in these cells (A. Yen and S. Varvayanis, unpublished data). A block of some other RA-effected cellular response thus presumably causes resistance. HL-60R−/D+ cells were cultured with either tazarotene plus an RXR-selective agonist, an RARα agonist plus an RXR agonist, tazarotene plus an RARα agonist plus an RXR agonist, 9-cis RA, or 1,25-dihydroxy vitamin D3. Figure 9 shows the percentage of differentiated cells and cells with $G_{1/0}$ DNA during treatment in each case tested. Tazarotene used in any of the tested combinations failed to induce differentiation or $G_0$ arrest, whereas 1,25-dihydroxy vitamin D3 caused both differentiation and $G_0$ arrest with kinetics characteristic of the wild-type parental cells reported previously. Tazarotene plus an RXR agonist was thus unable to overcome RA resistance

in these cells, consistent with the above-mentioned suggestion that tazarotene and an RARα agonist effect equivalent cellular responses.

## Discussion

The data are consistent with the suggestion that tazarotene, acting through RARβ in concert with an RXR agonist, causes ERK activation, wholesale conversion of hyperphosphorylated RB to the hypophosphorylated form, and consequential cell differentiation and $G_0$ cell cycle arrest. There are obvious caveats to this RARβ based interpretation. One cannot eliminate the possibility that the tazarotene is affecting cell physiology in some way that is independent of its action through RARβ but that mimics the necessary seminal process(es) attributed to RA or the combination of an RARα-selective agonist plus an RXR-selective agonist. One possible candidate process is the known ability of tazarotene and other retinoids to inhibit AP-1 activation (Nagpal et al., 1995). For example, tazarotene and other retinoids can block 12-O-tetradecanoylphorbol-13-acetate-induced AP-1 activation-dependent gene regulation in some cells. In the case of HL-60 cells treated with RA, the inhibition of AP-1 as a component of the machinery that propels the cell cycle and
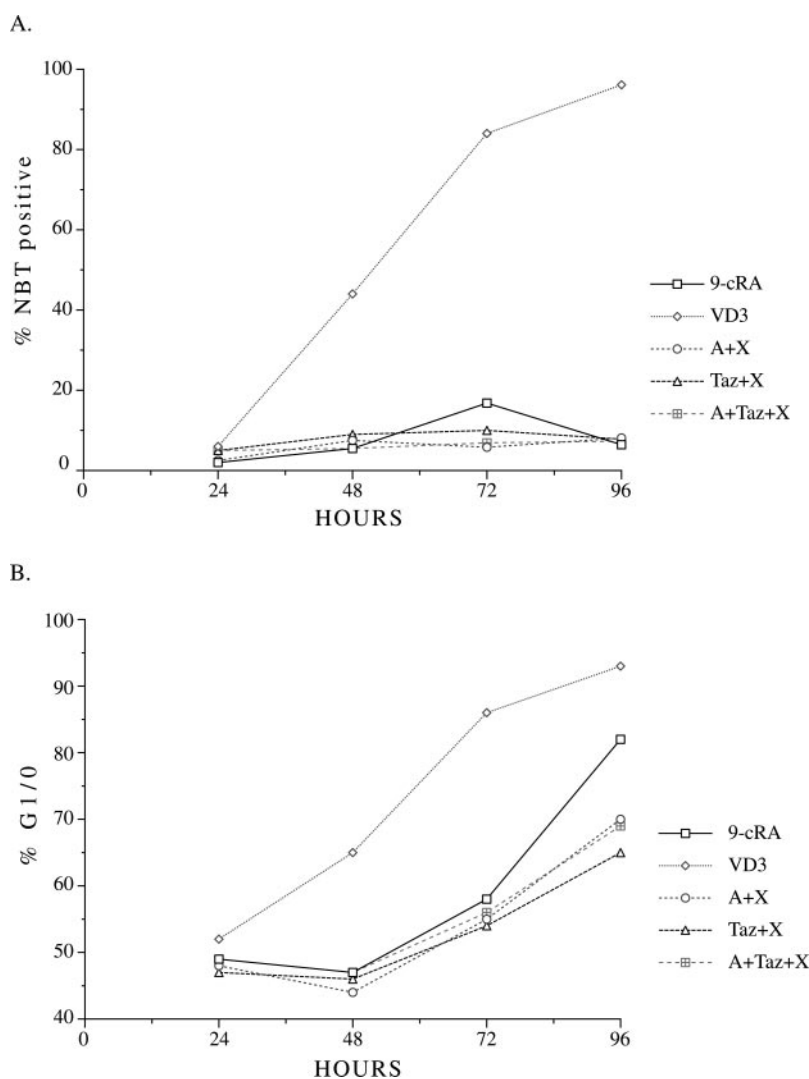


**Fig. 9.** Cell differentiation and cell cycle arrest after treating RA-resistant HL-60 cells, HL-60R−/D+, with RA, 1,25-dihydroxy vitamin D3, or combinations of tazarotene, RARα-selective agonist, and RXR-selective agonist. Differentiation (A) and cells with $G_{1/0}$ DNA (B) in HL-60R−/D+ cells treated with 9-cis retinoic acid (open square), 1,25-dihydroxy vitamin D3 (closed circle), RARα-selective agonist plus RXR-selective agonist (open circle), tazarotene plus RXR-selective agonist (triangle), RARα-selective agonist plus tazarotene plus RXR-selective agonist (closed square). Vertical axis, percent. Differentiation (A) and relative number of $G_{1/0}$ DNA cells (B) are as described in Fig. 5 legend. Horizontal axis, duration of treatment (hours).

TABLE 1

Dose response for induced percentage of NBT positive or percentage of $G_{1/0}$ at 72/96 h as a function of concentration of RAR$\alpha$ agonist or tazarotene for treatment by RAR$\alpha$ agonist + X or tazarotene + X where nonvarying RXR agonist (X) concentration is $10^{-6}$ M

The %$G_{1/0}$ hardly decreases at low RAR$\alpha$ agonist ($10^{-9}$ M) concentration, which is in contrast to low tazarotene concentration.

| | NBT (+) | | $G_{1/0}$ | |
| --- | --- | --- | --- | --- |
| Concentration | RAR$\alpha$ Agonist + X | Tazarotene + X | RAR$\alpha$ Agonist + X | Tazarotene + X |
| | [RAR$\alpha$ Agonist] | [Tazarotene] | [RAR$\alpha$ Agonist] | [Tazarotene] |
| | % | | | |
| $10^{-6}$ M | 84/94 | 71/96 | 76/85 | 69/85 |
| $10^{-7}$ M | 76/85 | 61/65 | 75/85 | 72/83 |
| $10^{-8}$ M | 63/84 | 32/60 | 74/83 | 64/76 |
| $10^{-9}$ M | 55/77 | 24/5 | 71/80 | 58/55 |
| Untreated control | 4/5 | 13/7 | 49/48 | 58/51 |

cell differentiation effects of RA has been largely discounted (Mollinedo et al., 1993; Kizaki et al., 1996). RA does not alter AP-1 activation in HL-60 cells (Mollinedo et al., 1993; Matikainen et al., 1994; Davis-Alison et al., 1997), nor does a retinoid that has AP-1 inhibitory activity without activating RAR or RXR transcriptional activity cause differentiation or growth arrest (Kizaki et al., 1996). There may be a variety of other yet occult processes that are also potential common targets of retinoids with antiproliferative or differentiation-inducing activity. Whereas these cannot be formally eliminated, the simplest rationalization of the present results is consistent with previous findings on RAR$\beta$'s tumor-suppressive effects, whereby tazarotene acting through RAR$\beta$ coadministered with an RXR agonist causes conversion of the proliferatively active HL-60 myeloblastic leukemia cells to a proliferatively quiescent, differentiated mature myeloid cell with the RB tumor suppressor interestingly acting as a potential downstream intermediary.

The sequential treatment data suggest that activation of RAR$\beta$, such as by tazarotene, and RAR$\alpha$, such as by the RAR$\alpha$ agonist, effect equivalent cellular responses during the early and late periods, leading to onset of differentiation and $G_0$ arrest. However, dose-response studies show that the effects of putative RAR$\alpha$ activation and RAR$\beta$ activation are probably not identical (Table 1). Comparing the dose-response data for RAR$\alpha$ agonist-induced differentiation and arrest and also tazarotene-induced differentiation and $G_0$ arrest showed that grossly diminishing RAR$\alpha$ agonist dose failed to grossly diminish induced cell cycle arrest in contrast to the diminishing responses in the other cases. The data are consistent with the suggestion that induced $G_0$ arrest may be more responsive to RAR$\alpha$ activation than RAR$\beta$ activation. Thus, whereas RAR$\beta$ and RAR$\alpha$ activation may cause equivalent cellular responses with respect to eliciting differentiation and $G_0$ arrest, this is not to say that they elicit identical responses. Tazarotene may thus provide an alternative to RAR$\alpha$ activation as a route to antineoplastic effects. The finding of potential antineoplastic activity for tazarotene is consistent with a recent report of the potential chemoprevention activity of tazarotene (So et al., 2004). Finally, a noteworthy implication of the studies presented here is that given these activities of tazarotene, then clinical administration of tazarotene in the presence of an RXR agonist has potential teratogenic activity.

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