Myeloid Differentiation and Retinoblastoma Phosphorylation Changes in HL-60 Cells Induced by Retinoic Acid Receptor- and Retinoid X Receptor-Selective Retinoic Acid Analogs

By S. Carroll Brooks III, Sonja Kazmer, Arthur A. Levin, and Andrew Yen

The ability of subtypes of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) singly and in combination to elicit myeloid differentiation, G_{10} specific growth arrest, and retinoblastoma (RB) tumor suppressor protein dephosphorylation was determined in the human myeloblastic leukemia cell line HL-60 using subtype-selective retinoic acid (RA) analogs. RA analogs that selectively bind only to RARs (Am580 and/or TTNPB) or to RXRs (Ro 25-6603, SR11237, and/or SR11234) did not elicit the above-mentioned three cellular responses. In contrast, simultaneous treatment with both an RAR-selective ligand (Am580 or TTNPB) and an RXR-selective ligand (Ro 25-6603, SR11237, or SR11234) induced all three cellular processes. An RAR-selective ligand used with an RXR-selective ligand generated the same responses as did all-trans RA or 9-cis RA, which affect both families of receptors, suggesting an important role for RARα among RAR subtypes in eliciting cellular response. Consistent with this finding, the RARα antagonist, Ro 41-5253, reduced the level of the cellular responses elicited by treatment with an RARα-selective ligand plus RXR-selective ligand. The coupling of the shift of RA to its hypophosphorylated form with G_{10} arrest and differentiation in response to ligands is consistent with a possible role of RA as a downstream target or effector of RARα and RXR in combination.

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From the Cancer Biology Laboratories, the Department of Pathology, Cornell University, Ithaca, NY; and the Department of Toxicology and Pathology, Hoffmann-LaRoche Inc, Nutley, NJ.

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Address reprint requests to Andrew Yen, PhD, Cancer Biology Laboratories, Department of Pathology, Cornell University, Ithaca, NY 14853.

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Allenby, unpublished observations), 4-[2-(5,5,8,8-Tetramethyl-5,6,7,8-tetrahydro-naphthalen-2-yl)-[1,3]dioxolan-2-yl]-benzoic acid (SR11237), 34-4-[2-(5,5,8,8-Tetramethyl-5,6,7,8-tetrahydro-naphthalen-2-yl)-[1,3]dithiolan-2-yl]-benzoic acid (SR11234), 35 and (all-E)-6-Bromo-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclo-hexen-1-y l)-2,4,6,8-non-uteroenoic acid (Ro 25-5802; Levin and Allenby, unpublished observations), selectively activate RXRs. These RXR-selective ligands provide a means of testing what receptors, among RARs and RXRs, are needed to induce myeloid differentiation, to induce G_{iso} growth arrest, and to change RB expression in HL-60 cells. These studies also test the coupling of RB hypophosphorylation to growth arrest or differentiation.

MATERIALS AND METHODS

Cell culture and treatments. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Intergen Co, Purchase, NY) and incubated at 37°C in a humidified atmosphere of 5% CO₂, as described previously. Stock cultures were maintained at constant exponential growth. Cultures were seeded at 0.2 × 10⁶ and 0.1 × 10⁶ cells/mL every 2 to 3 days, respectively. The cells show no lag time upon seeding in fresh medium, but continue to grow with a generation time of approximately 20 hours. Control cells can grow to greater than 3 × 10⁶ cells/mL, but exhibit nutritional deprivation effects at high cell density. Other sublines also have approximately 20-hour generation times and high saturation densities. 35

The RA analogs Am580, TTNPB, Ro 25-6603, SR11237, SR11234, Ro 25-5802, CD437, and Ro 41-5253 were synthesized by the Department of Medicinal Chemistry, Hoffmann La Roche (Nutley, NJ and Basel, Switzerland). All retinoids were all dissolved in 100% ethanol to achieve final stock concentrations of 1 pmoVL. Cell counts taken at this time ensured that at least 90% of the cells were retained in the culture. The experiments shown represent either the average or typical response to treatment among three repeats.

Differentiation assay. HL-60 functional differentiation was assayed by measuring oxidative metabolism on a per cell basis via superoxide reduction of nitroblue tetrazolium (NBT) to formazan, a hallmark of mature myeloid cells, as described previously. 3 Between 1 and 2 × 10⁶ cells were suspended in 100 to 200 μL of the NBT/phorbol myristate acetate (PMA) solution (2.4 mM NBT and 0.32 mM PMA). Cells were incubated in this NBT/PMA solution for 20 minutes at 37°C with intermittent agitation. The cells were scored on a hemocytometer. A purple cytoplasmic precipitate defined a cell that was positive for inducible oxidative metabolism. A clear cell signified a negative result. At least 200 cells were scored per assay.

Flow cytometry. The cell cycle analysis was performed using propidium iodide stained cells analyzed on a multiparameter dual-laser flow cytometer with an argon ion laser delivering excitation at 488 nm (Coulter Electronics, Hialeah, FL). At indicated times, 0.5 × 10⁶ cells were centrifuged to a pellet and resuspended in 0.5 mL propidium iodide solution. 2 The suspension was then stored at 4°C until analyzed, as described previously. 3 The percentage of cells with a G_{iso} DNA complement was determined from a DNA histogram by region integration using onboard data analysis routines.

Western blot analysis. Western analysis of the RB protein was performed as described previously. 28, 36 At indicated times, 1.0 × 10⁶ cells were centrifuged to a pellet and the supernatant was aspirated off. The pellet was resuspended in 100 μL phosphate-buffered saline (PBS) to which 900 μL of 100% methanol was added to fix the cells in solution before the cells were stored at −80°C. For analysis, the cells were centrifuged to a pellet, the supernatant was once again aspirated off, and the cells were resuspended in lysis buffer as described previously. 37 The RB protein was resolved on a 6% sodium dodecyl sulfate polyacrylamide gel (run at 280 V for 2 hours), transferred to a nitrocellulose membrane by electroblothing (at 200 V for 1 hour), and detected with a murine IgG; monoclonal primary antibody (catalogue no. 10061; Triton Diagnostics, Alameda, CA) that recognizes both the unphosphorylated and phosphorylated RB protein. An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) was used to produce an image of the RB protein from the nitrocellulose blot on the autoradiographic film. A scanning densitometer (Joyce-Loebl, Ltd, Gateshead, Tyne on Wear, UK) was used to generate densitometric scans of the Western blot autoradiograms. The ordinate shows the absorbance that is proportional to RB band intensity on the autoradiogram, and the abscissa shows the position of the band along 20 mm of the Western blot autoradiogram oriented with the bottom of the gel at the right. The broad hyperphosphorylated band, which is about 110 kD, appears closer to the origin of the graph and is the prominent peak at the 0-hour and each of the 24-hour time points. The hypophosphorylated band appears to the right on the x-axis in the later time point scans. The intensity of the bands varies from series to series due to the fact that the series were on different gels, resulting in different autoradiograms of varying intensity. Because the relative proportion of the two forms of RB protein is being compared between treatments and not the level of expression, this does not affect the presented analysis.

Retinoid binding assays. The nucleosol fraction of COS-1 cells transiently transfected with expression plasmids containing the cDNA for the RAR and RXR subtypes were used as a source of binding activity. Three days after transfection, nuclei from transfected Cos-1 cells were isolated and lysed in an assay buffer. This yielded a nucleosol rich in an RAR or RXR subtype. The receptor binding assay used an unlabeled retinoid analog to displace ³H-9-cis RA (9CRA; 10 nmoVL) or ³H-all-trans RA (ATRA; 5 nmoVL) from an RAR or RXR subtype, respectively. The lysate was incubated with radioligand and unlabeled competing ligand for 3 hours to allow for equilibration. Bound ligand was separated from free ligand by gel filtration using PD10 (Pharmacia, Uppsala, Sweden) columns. 28 The assay was performed under amber light to minimize photoisomerization of the retinoid analogs. Using a series of competitor concentrations, an inhibitory concentration curve was generated. The inhibitory concentration to displace 50% of the tritiated ligand (IC₅₀) was determined, as described previously. 38 The IC₅₀ was calculated for each analog with each receptor subtype using a four-parameter logistic equation (Allfit; NIH, Laboratory of Theoretical and Physical Biology, NICHD,
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Table 1. Competition Binding IC<sub>50</sub> (Nanomoles per Liter) Values for RA Analogs

<table>
<thead>
<tr>
<th></th>
<th>mRARα</th>
<th>mRARβ</th>
<th>mRARγ</th>
<th>mRXRα</th>
<th>mRXRβ</th>
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<tr>
<td>Aα</td>
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<td>17,000</td>
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<tr>
<td>A</td>
<td>15</td>
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<td>7</td>
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<td></td>
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<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>X2</td>
<td>*</td>
<td></td>
<td>*</td>
<td>1,500</td>
<td>3,000</td>
<td>400</td>
</tr>
<tr>
<td>X3</td>
<td>*</td>
<td></td>
<td>*</td>
<td>9,700</td>
<td>6,800</td>
<td>8,500</td>
</tr>
<tr>
<td>X4</td>
<td>3,400</td>
<td>6,600</td>
<td>12,000</td>
<td>40</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td>9CRA</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Binding assays were based on competing tritiated ATRA (2.5 to 5 nmol/L) from RARs or tritiated 9CRA (5 to 10 nmol/L) from RXRs (Aα, Am580; A, TTNPB; X1, Ro 25-6603; X2, SR11237; X3, SR11234; and X4, Ro 25-5802). The IC<sub>50</sub> represents the concentration of cold analog required to compete off 50% of the tritiated ATRA or 9CRA.

Abbreviations: *, an IC<sub>50</sub> greater than 10,000 nmol/L; —, an IC<sub>50</sub> greater than 25,000 nmol/L.

Bethesda, MD. The RA analogs were a generous gift of Hoffmann-LaRoche, Inc (Nutley, NJ).

RESULTS

HL-60 cells were treated with RA analogs that had selective affinities distinguishing the RARs or RXRs as a family (>10-fold difference) or the specific subtypes of RARs or RXRs (>20-fold difference). The structures of the RA analogs and their binding selectivities for the different RAR and RXR subtypes are shown in Table 1 and Fig 1 (Aα, Am580; A, TTNPB; X1, Ro 25-6603; X2, SR11237; X3, SR11234; and X4, Ro 25-5802).<sup>25,31-33</sup> The binding selectivities are described by the IC<sub>50</sub>, which represents the amount of retinoid needed to inhibit binding of tritiated ligands by 50%. A lower IC<sub>50</sub> thus indicated a greater affinity of the analog for the receptor. Aα and A have lower IC<sub>50</sub> for the RAR subfamily than for the RXR subfamily. They thus have preferential affinity for RAR versus RXR and are therefore RAR-selective. Aα shows a 20-fold lower IC<sub>50</sub> for RARα than for RARβ and is therefore RARα-selective. Unlike Aα, A does not discriminate between RARα and RARβ, binding each with nearly the same affinity. The importance of RARβ ligand-binding for eliciting HL-60 cell differentiation could thus be tested with these ligands. Four ligands had RXR selectivity, ie, X1, X2, X3, and X4. X1 competes with 9CRA for binding to the RXRs as a family better than X2 or X3, and X2 competes with 9CRA for binding to RXRα better than X3. This difference enabled the importance of the binding affinity of the RXR-selective ligand in differentiation to be tested. The RXR-selective analog X4 retains some affinity for the RARs and was useful as a semiselective analog. The observed binding is consistent with previous transactivation studies performed in vitro that showed the selective activation of RARα by Aα, RARs by A, and RXRs by X2 and X3.<sup>33,40,41</sup>

A typical ATRA-treated HL-60 cell population showed onset of differentiation at 48 hours, when the percentage of cells positive for inducible oxidative metabolism increased, the percentage of cells with a G1/0 DNA content increased, the growth rate decreased, and the percentage of RB protein that was hypophosphorylated increased relative to control cells. By 96 hours, the differentiating population of cells was more than 90% positive for inducible oxidative metabolism and more than 90% had a G1/0 DNA content. The cell density was less than 1.5 × 10<sup>6</sup> cells/mL and more than 95% of the RB was hypophosphorylated.<sup>28,37</sup> These levels indicate that ATRA is an effective inducer of the myeloid differentiation of HL-60 cells. ATRA can be converted into 9CRA by isomerization. The 9CRA isomer can bind both RARs and RXRs.
RXRs.\textsuperscript{38,42} Figure 2A shows the above responses of a 9CRA-treated population of HL-60s, compared with an untreated control population. The data show that 9CRA effectively induces myeloid differentiation, G\textsubscript{10}-specific growth arrest, and RB dephosphorylation (Fig 2B). Untreated HL-60 cells did not exhibit these changes, as previously shown, except for effects due to high cell density and nutritional deprivation that become apparent at 96 hours.\textsuperscript{38,43} The increase in the %G\textsubscript{1} and sudden decrease in the cell density for the control population at 96 hours is attributable to this nutritional deprivation. Cell densities greater than 3 \times 10\textsuperscript{6} cells/mL were observed in these control cultures undergoing nutritional deprivation.

To ascertain whether HL-60 cells could differentiate in the presence of a ligand that selectively bound the RARs or RXRs, each of the six analogs were used singly to treat HL-60 cells. It was found none of the two RAR-selective (A\textalpha or A) or three of the RXR-selective (X\textsubscript{1}, X\textsubscript{2}, or X\textsubscript{3}) ligands alone caused any significant increase in the percentage of cells positive for inducible oxidative metabolism, increase in the percentage of cells with a G\textsubscript{10} DNA content, decrease in cellular proliferation, or increase in the percentage of hypophosphorylated RB (data not shown). Cells treated with each of these single analogs were indistinguishable from untreated cells (Fig 2A and B). Simultaneous treatment with combinations of two RAR- (A\textalpha or A) or two RXR-selective ligands (X\textsubscript{1}+X\textsubscript{2}, X\textsubscript{1}+X\textsubscript{3}, or X\textsubscript{2}+X\textsubscript{3}) also failed to induce differentiative changes in any of the above-mentioned indices (data not shown). This finding indicated that treatment with an analog or analogs (at 1 \mu mol/L) that bound selectively to only one of the two subfamilies of retinoid receptors could not induce differentiation. In contrast, treatment with an RXR-selective ligand that retains some RAR binding, X\textalpha, did cause some response measured by these cellular processes as well as RB hypophosphorylation.

96 hours, cells treated with X\textsubscript{4} were 73\% positive for inducible oxidative metabolism, 79\% had G\textsubscript{10} DNA content, and 59\% of the RB was hypophosphorylated. Growth measured by cell density slightly exceeded control cells (3.29 \times 10\textsuperscript{6} cells/mL). Because HL-60 cells do not express RAR\textgamma, the RAR\textgamma-selective ligand, CD437, was used to test for any non-receptor-mediated effects.\textsuperscript{44} HL-60 cells treated with CD437 showed no apparent effects on RB, cell proliferation, or differentiation compared with control cells (data not shown).

Each of the treatments that combined an RAR-selective with an RXR-selective analog was effective at inducing myeloid differentiation, G\textsubscript{10}-specific growth arrest, a decrease in proliferation, and a shift to the hypophosphorylated form of RB. The results of treating HL-60 cells with A\textalpha+X\textsubscript{1}, A\textalpha+X\textsubscript{2}, A\textalpha+X\textsubscript{3}, or A\textalpha+X\textsubscript{4} are shown in Fig 3A and B. The percentage of cells positive for inducible oxidative metabolism, the percentage of cells with a G\textsubscript{10} DNA content, the cell density, and the percentage of hypophosphorylated RB are shown. This treatment induced changes in each of these parameters. The onset of change in each case was seen at 48 hours. At 96 hours, for the A\textalpha+X\textsubscript{1}, A\textalpha+X\textsubscript{2}, A\textalpha+X\textsubscript{3}, or A\textalpha+X\textsubscript{4} cases, 92\%, 93\%, 75\%, and 91\% of the treated cells were positive for inducible oxidative metabolism and 97\%, 91\%, 92\%, and 96\% had a G\textsubscript{10} DNA content, respectively. The cell density was 1.30 \times 10\textsuperscript{6}, 0.77 \times 10\textsuperscript{6}, 1.60 \times 10\textsuperscript{6}, and 1.16 \times 10\textsuperscript{6} cells/mL, and 98\%, 99\%, 78\%, and 99\% of the RB was hypophosphorylated, respectively. Each of the parameters measured showed that treatments with A\textalpha and any of the RXR-selective ligands in combination induced HL-60 myeloid differentiation.

In contrast to A\textalpha, A selectively bindsRAR\textalpha, -\beta, and -\gamma. The results of treating with A+X\textsubscript{1}, +X\textsubscript{2}, +X\textsubscript{3}, or +X\textsubscript{4} are
Fig 3. RARα- and RXR-selective analogs combined. (A) The percentage of RA analog-treated HL-60 cells and untreated controls (Aα, Am580; X1, Ro 25-6603; X2, SR11237; X3, SR11234; and X4, Ro 25-5802; each retinoid is at a concentration of 1 μmol/L) that are functionally differentiated as measured by inducible oxidative metabolism (top panel), the percentage of cells with G1 DNA as determined by flow cytometry (middle panel), or the cell density as determined by hemocytometric counts (bottom panel), as a function of time. (B) Densitometric scans of Western blots of HL-60 cells treated with combinations of RA analogs. The bottom of the gel is to the right, and the horizontal axis represents 20 mm on the gel. The left-most panel is a 0-hour control scan that shows the peak representing the hyperphosphorylated RB protein. The four subsequent panels show scans for each of the treatments at 24, 48, 72, and 96 hours. The right peak is the faster migrating hypophosphorylated RB protein. The percentage of hypophosphorylated RB is denoted for each curve.

shown in Fig 4A and B. The results of these treatments using A were similar to those using Aα. In contrast, treatments with 6-(3-(1-Adamantyl)-4-hydroxyphenyl)-2-naphthoic acid (CD437), an RARγ-selective ligand, in combination with X1 or X2 failed to induce differentiation (data not shown). This is consistent with the known lack of RARγ in HL-60 cells.

To determine if the action of RAR- or RXR-selective analogs could segregate specifically with early or late events in the process of differentiation, HL-60 cells were treated with an RAR or RXR selective ligand for 24 hours, at which time the cells were washed and subsequently treated with a ligand that would selectively bind the other receptor subtype. Each of the treatments with an RAR and then an RXR ligand induced differentiation with slower kinetics, whereas each of the treatments with an RXR and then an RAR ligand were ineffective. The results of treating with Aα and then X1, X2, X3, or X4 (denoted Aα+Xn) are shown in Fig 5A and B. The onset of differentiation was not seen until 72 hours. This was 24 hours later than the onset of differentiation when RAR- and RXR-selective analogs were simultaneously used (Figs 2A and B and 3A and B). At 96 hours, the values for each of the cellular responses also reflected the 24-hour delay seen at the onset. The cells were 77%, 73%, 62%, and 72%
positive for inducible oxidative metabolism, and 83%, 86%, 79% and 84% had a G1 DNA complement. The cells were at a density of \(4.17 \times 10^6\), \(2.71 \times 10^6\), \(3.13 \times 10^6\), and \(3.02 \times 10^6\) cells/mL, and 73%, 80%, 62%, and 66% of the RB protein was hypophosphorylated, respectively. Treatments with an RAR-selective ligand followed by an RXR-selective ligand (denoted A+X₁) also showed onset of each of the four characterized responses at 72 hours, as shown in Figs 6A and B. The responses are consistent with the need for 48 hours of simultaneous exposure to both an RAR- and RXR-selective ligand for onset of differentiation, as described above. The differences in growth arrest and cell density between the RAR→RXR and RXR→RAR treatments, although not gross, were repeatable in three assays and most apparent in each of the differentiation assays. The relative affinity of X₁ and X₂ versus X₃ binding is also consistent with the relative induced differentiation. For example, the A+X₃ sequence of analogs had the weakest binding and the weakest effects. Thus, the sequence of early and late events did not resolve as RAR- or RXR-selective events in sequence.

An RARα antagonist was added to the ligand combination treatments to evaluate the involvement of RARα in the myeloid differentiation of HL-60 cells. In the Aα+X₃ treatment,
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Fig 5. RARα- and RXR-selective analogs in sequence. (A) The percentage of RA analog-treated HL-60 cells and untreated controls (Aα, Am580; X1, Ro 25-6803; X2, SR11237; X3, SR112344; and X4, Ro 25-6802; each retinoid is at a concentration of 1 μmol/L) that are functionally differentiated as measured by inducible oxidative metabolism (top panel), the percentage of cells with G1 DNA as determined by flow cytometry (middle panel), or the cell density as determined by hemocytometric counts (bottom panel), as a function of time. All treatments were washed of the first analog at the 24-hour time point and then immediately treated with the second analog. (B) Densitometric scans of Western blots of HL-60 cells treated with RA analogs. The bottom of the gel is to the right, and the horizontal axis represents 20 mm on the gel. The left-most panel is a 0-hour control scan representing the hyperphosphorylated RB protein. The four subsequent panels show scans for each of the treatments at 24, 48, 72, and 96 hours. The right peak is the faster migrating hypophosphorylated RB protein. The percentage of hypophosphorylated RB is denoted for each curve.

reducing the concentration of Aα from 1 μmol/L to 10 nmol/L was as effective at inducing differentiation, growth arrest, and G1 arrest as 1 μmol/L (Table 2 and Fig 6). When 1 μmol/L RARα antagonist is added to the Aα (10 nmol/L) +X2 treatment, the RARα antagonist reduced the induced response. This was consistently apparent by four criteria. By 72 hours, there was a decrease of 17% in the number of cells that were positive for inducible oxidative metabolism, a decrease of 7% in the number of cells that had a G1 DNA content, an increase of 51% in the cell density, and a decrease of 24% in hypophosphorylated RB protein (Table 2 and Fig 7). When the concentration of Aα was increased 100-fold to 1 μmol/L, creating an equimolar concentration of the RARα antagonist and Aα of 1 μmol/L, the effect of the antagonist was neutralized (data not shown; Fig 6).

DISCUSSION

We tested the effectiveness of different RAR and RXR subtype-selective ligands, individually and in combination, to effect changes in RB expression/phosphorylation, cellular proliferation, and cellular differentiation. Because 9CRA binds all RARs and RXRs and ATRA can be metabolized to 9CRA, RAR-mediated effects cannot be segregated from RXR-mediated effects in treatments with 9CRA or ATRA. To determine which receptors were responsible for the induction of differentiation, we treated cells with
When two ligands, one selective for an RAR and the other for an RXR, were applied in combination, myeloid differentiation was enough to induce differentiation if each of the two RAR-selective analogs (Aα+A), or two RXR-selective differentiations. Not even twice the total retinoid concentration of unphosphorylated RB was evident relative to controls. When treatments consisted of a sequence of retinoids, the efficacy of the Aα+X1 treatment combination by adding the RARα antagonist at a 100:1 ratio with Aα. These experiments showed that neither an RAR-selective nor an RXR-selective ligand alone could induce myeloid differentiation. Not even twice the total retinoid concentration was enough to induce differentiation if each of the two ligands were selective to either RARs or RXRs alone, as in the Aα+A and X2, X3, or X4 treatments. If the selectivity of the ligand can be extrapolated to the responsive genes that the RARs and RXRs target, then this suggested that neither the RAR-mediated pathways nor the RXR-mediated pathways are sufficient to elicit the differentiation program in HL-60 cells.

In comparing the array of single, combinatorial, and sequential ligand treatments studied here, the treatment that caused the greatest differentiation was that in which Aα, the analog selective for RARα, was administered in combination with X1, X3, or X4, the RXR-selective analogs. The Aα+X1, X3, or X4 and A+X1, X3, or X4 treatments were similarly effective, showing that further restricting ligand binding among RARs to RARα did not appreciably diminish the level of induced myeloid differentiation, growth arrest, or RB dephosphorylation. This latter finding may relate to the composition of receptors in HL-60 cells that express more RARα protein than RARβ protein. These data are consistent with the hypothesis that RARα- and RXR-responsive genes are sufficient to elicit the differentiation program in HL-60 cells. This may reflect activation by this heterodimer, but simultaneous contributions from RARα-RARx, RXR-RXR, and RARα-RXR responsive genes might also mediate HL-60 myeloid differentiation. The inhibition of Aα+X2 effects by the RARα antagonist, Ro 41-5253, is also consistent with the potentially prominent role of RARα among RARs.

Consistent with the data above, it is interesting to see that X4, a compound with strong binding to the RXRs and weak but significant binding to the RARs, still retains some HL-60 differentiation activity. When X2 was combined with Aα or A, an increase was evident in each of the cellular responses measured. This suggested that the RAR binding affinity of X4 was not enough to elicit a full response in our assay system, but, when supported by the RAR-binding affinity of Aα or A, the response was elevated.

Our results provide a partial rationalization of previous results with HL-60 cells treated with TTAB (which activates RARs and RXRs) and SR11217 (which activates RXRs).41 TTAB, but not the SR11217, caused inhibition of clonal growth or differentiation, but a treatment with TTAB and SR11217 did not synergistically induce differentiation to a greater degree than TTAB alone.41 These retinoids may not have acted synergistically due to the fact that TTAB activates both RARs and RXRs and thus alone adequately induces myeloid differentiation. Synergy might have been seen had an RAR-selective analog such as Aα, which selects for RARα and does not induce differentiation alone, was used in combination with SR11217.

Treatments with sequences of analogs were used to observe whether the early and late events of HL-60 cellular differentiation could be driven by analogs selective for RARs or RXRs. The treatment sequence of an RAR-selective ligand followed 24 hours later by an RXR-selective ligand induced onset of differentiation at 72 hours, whereas an RXR-selective ligand followed by an RAR-selective ligand failed to cause any significant level of differentiation above controls. In contrast to the simultaneous treatments with combinations of ligands, which showed onset of differentiation at 48 hours, the effects of the treatments of sequences of ligands are not apparent until 72 hours. Thus, RAR- and RXR-selective RA analogs apparently had to be simultaneously present for 48 hours to affect differentiation. Neither early nor late events in the cascade leading to myeloid differentiation could be solely induced by RARs or RXRs alone.

Our results on the effects of Aα and the RARα antagonist relate to a previous report of nearly complete antagonism of Aα-induced HL-60 cellular differentiation by Ro 41-5253.27 Those results showed that treatment of HL-60 cells with 10 nmol/L Aα alone induced some differentiation. The addition of 1 μmol/L Ro 41-5253 reduced the level of differentiation by nearly 75%. Although the conclusions drawn from these studies diverge from those presented here, the data are not inconsistent.27 The difference in conclusions stems from two causes. One cause is that the two studies did not use the same assay for assessing differentiation. The previous studies spectrophotometrically scored the amount of superoxide produced by the cell population as a whole, whereas we microscopically scored the fraction of cells expressing induc-

Table 2. RARα Antagonist Reduction of Differentiative Capacity of RARα-Selective Ligand

<table>
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<tr>
<th>Treatment</th>
<th>% Differentiated</th>
<th>% G1 Growth Arrest</th>
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<td>2.6</td>
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<td>78</td>
<td>1.68</td>
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<td>71</td>
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</table>

Aα, Am580; X2, SR11237. All data are from samples collected at the 72-hour time point.
ible superoxide. The other cause is the amount of inducible superoxide response that qualified the responding population to be considered differentiated. In the previous study, differentiation was assayed by the amount of nitroblue tetrazolium reduced by a whole population of cells measured by spectrophotometric absorption. This does not measure the percentage of cells that have differentiated but rather the superoxide production of the population as a whole. Using this assay, 10 nmol/L AM580 was reported to induce differentiation. A concentration of 100 nmol/L induced a greater observed absorbance (as shown in their Fig 3). The absorbance observed at 100 nmol/L was approximately 4 times their background. In our hands, background is typically 5% differentiated cells. Thus, their observed absorbance converts to approximately 20% differentiation by our assay. This is consistent with our data. But, relative to the 90% to 100% differentiated cells that we observed using 9CRA or combinations of RAR- and RXR-selective ligands, the response to Am580 alone was both very weak in extent and late in timing. In our studies, this lack of significant differentiation was correlated with a lack of G1 arrest or inhibition of cell growth, as well as of dephosphorylation of RB. Thus, we considered

**Fig 6.** RAR- and RXR-selective analogs in sequence. (A) The percentage of RA analog-treated HL-60 cells and untreated controls (A, TTNPB; X, Ro 25-6603; X, SR11237; X, SR112344; and X, Ro25-6802; each retinoid is at a concentration of 1 μmol/L) that are functionally differentiated as measured by inducible oxidative metabolism (top panel), the percentage of cells with G1 DNA as determined by flow cytometry (middle panel), or the cell density as determined by hemocytometric counts (bottom panel), as a function of time. All treatments were washed of the first analog at the 24-hour time point and then immediately treated with the second analog. (B) Densitometric scans of Western blots of HL-60 cells treated with RA analogs. The bottom of the gel is to the right, and the horizontal axis represents 20 mm on the gel. The left-most panel is a 0-hour control scan representing the hyperphosphorylated RB protein. The four subsequent panels show scans for each of the treatments at 24, 48, 72, and 96 hours. The right peak is the faster migrating hypophosphorylated RB protein. The percentage of hypophosphorylated RB is denoted for each curve.
AM580 ineffective at inducing differentiation. In contrast, the previous study considered this small amount of differentiation induced as nevertheless evidence of differentiation but without regard to the number of cells responding. Thus, the previous data are consistent with the presently observed low level of differentiation at late times induced by Aa once differences in the assays are accounted for.

The treatments that did not elicit differentiation, involving analogs that selected for only one subclass of receptors, also did not cause any change in the percentage of the RB protein that was hypophosphorylated. Treatments that did induce differentiation also caused changes in the percentage of hypophosphorylated RB protein. Thus, there was an apparent coupling between elicited effects on RB and on cell differentiation and on growth arrest. Each of the treatments that induced myeloid differentiation used ligands that selected for RARα and RXR in combination. Thus, both RAR- and RXR-mediated pathways are required for the induction of changes in RB phosphorylation, differentiation, and growth arrest. Treatments with RAR- or RXR-selective ligands individually were much less effective than combinations in eliciting changes in RB phosphorylation or differentiation. Taken together, the data suggest that one of the downstream retinoid-mediated events of the differentiation of HL-60 cells is the downregulation and hypophosphorylation of RB driven by RAR and RXR activation. RB may thus be a molecular mediator of the retinoid-induced cellular response of HL-60 myeloid differentiation.

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Myeloid differentiation and retinoblastoma phosphorylation changes in HL-60 cells induced by retinoic acid receptor- and retinoid X receptor-selective retinoic acid analogs

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