Activation of Three Distinct RXR/RAR Heterodimers Induces Growth Arrest and Differentiation of Neuroblastoma Cells

Giuseppe Giannini, Marcia I. Dawson, Xiao-kun Zhang, and Carol J. Thiele

From the Cell and Molecular Biology Section, Pediatric Oncology Branch, NCI, National Institutes of Health, Bethesda, Maryland 20892-1928; Retinoid Program, SRI International, Menlo Park, California 94025, and The Burnham Institute, La Jolla, California 92037

Naturally occurring retinoids, like all-trans retinoic acid and 9-cis retinoic acid, are known to affect proliferation and differentiation of sensitive neuroblastoma cell lines. Cellular responsiveness to retinoic acid depends on its interaction with two distinct classes of receptors, the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs). Both receptor classes have three different subtypes (RARα, RARβ, and RARγ and RXRα, RXRβ, and RXRγ) that act as ligand-dependent transcription factors. To examine the involvement of the different receptor classes and subtypes in the biological responses of neuroblastoma cells to retinoids, we analyzed the effects of a panel of receptor-selective retinoids on cell growth, differentiation, and gene expression on in vitro cultured KCNR cells. Any association of per se inactive RAR-selective with RAR-selective ligands efficiently regulates growth inhibition, differentiation (neurite extension), and expression of RARβ, TrkB, and N-myc. SR11383 alone, a very potent retinoid, entirely reproduces the pattern of biological responses induced by naturally occurring retinoids. In contrast to other tumor cell lines, the growth of neuroblastoma cell lines is not altered using AP1-antagonistic retinoids. These studies establish the possibility that three distinct RAR/RAR heterodimers mediate the effects of retinoids on neuroblastoma cells through an AP1 antagonism-independent mechanism.

At the transcriptional level, the effects of retinoids are mediated by two classes of ligand-dependent transcriptional activators: the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs), which are members of the nonsteroid nuclear hormone receptor superfamily (4). Both receptor classes have three different subtypes (RARα, RARβ, and RARγ and RXRα, RXRβ, and RXRγ) that encoded by different genes, each of which can give rise to different isoforms (4, 5). Naturally occurring retinoids, like all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9CRA) have different affinities for these receptor classes. ATRA only binds to RARs, whereas 9CRA binds to both RARs and RXRs (6–9). However, ATRA can activate RXRs presumably through its isomerization to 9CRA, which is known to occur in living cells (9). Ligand-activated receptors undergo conformational changes (10, 11), which increase their binding affinity for specific RA-responsive elements (RAREs) (12). This leads to selective transcriptional activation of specific target genes. Retinoid receptors may also regulate gene expression by functionally interacting with other transcription factors, i.e. AP-1 (13–15) by a mechanism that appears to play a major role in the growth inhibition of several cancer or transformed cell lines (16, 17).

Although RXR/RXR homodimers can efficiently activate transcription of certain promoters (18), several studies have demonstrated that the RAR/RAR heterodimer is the transactivator responsible for transducing the signal in response to retinoids (19–22). RXRs also interact with other members of the nuclear hormone receptor family like thyroid hormone receptors, the vitamin D₃ receptor, the peroxisome proliferator-activated receptor, and several orphan receptors (23).

A certain degree of functional redundancy among the various RAR/RAR heterodimers in the activation of complex genetic programs by retinoids is suggested by the absence of gross morphological alterations observed in single-receptor knockout mice (4, 5) and the analysis of RAR-null F9 embryonal carcinoma cells (24). However, the distinct pattern of expression of the different RARs and RXRs in both developing and adult organisms suggests their involvement in at least partially distinct physiological functions (25, 26). RARα is ubiquitously expressed both during development and in adult tissues (25, 26). RARβ is apparently more involved in the differentiation of certain epithelia and in the ontogenesis of the nervous system (25, 26). The preferential expression of RARγ in several developing mesenchymes and the subsequent restriction of its expression to cartilage and squamous keratinizing epithelia (25, 27) indicates its prominent role in morphogenesis, condrogenesis, and epithelial cell differentiation. In defined cell culture systems, the RAR principally involved in the activation of complex biological responses to retinoids appears to be different. RARγ is the predominant class of receptor expressed in F9 cells, where it is necessary at least for the induction of differentiation by retinoids (28). RARα, but not RARβ, can fully
substitute for RARγ when overexpressed in RARγ null cells (24, 29). RARα is the predominant subtype in P19 cells (29), and it also seems to play a major role in the control of growth and differentiation of leukemia cells (30–32).

Upon treatment with RA, several in vitro cultured NB cell lines undergo growth inhibition and a morphological differentiation that is characterized by the extension of neurites. These phenomena are associated with and perhaps dependent on the regulated expression of several genes. In particular, the inhibition of N-myc expression (33–35), the induction of the neurotrophin receptor TrkB (36, 37), and the induction of RARβ (38, 39) are thought to play a fundamental role in the induction of both morphological changes and growth inhibition by retinoids. Although the effects of retinoids on NB have long been known, very little information is available on the relative contribution of the specific RAR and RXR classes and subtypes. We have addressed this question by analyzing the effects of a panel of receptor-selective retinoids on the biology of KCNR NB cells. We show here that treatment with any association of an RAR-selective ligand and an RXR-selective ligand induced inhibition of cell growth, differentiation, and regulated gene expression in KCNR cells. Therefore we suggest that, in combination with RARα, the three different subtypes of RAR contribute to NB cell responses to retinoids. Growth inhibition in particular is mediated by a mechanism independent of AP-1 antagonism. SR11383, an exceptionally potent retinoid, completely repro-
duced the pattern of biological responses to ATRA. Moreover, in combination with an RXRα-selective ligand, it induced growth inhibition and differentiation of NB cells more efficiently than ATRA and 9CRA.

EXPERIMENTAL PROCEDURES

Cell Culture—The SMS-KCNR (KCNR) cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C with 5% CO2. For stimulation experiments, cells were seeded at a density of 140 cells/mm2 in medium containing charcoal-stripped FBS. After 24 h, cells were treated with the different retinoids dissolved in MeSO or MeSO alone as a solvent control. After 4 days, representative fields from each dish were photographed with a Nikon Diaphot inverted microscope to record morphological differentiation. For 3H-thymidine incorporation experiments, KCNR cells were seeded at 105 cells/well in 96-well plates in medium containing charcoal-stripped FBS. After 24 h, the appropriate analogous or solvent control were added to quadruplicate wells, and the plates were incubated for 4 days under standard conditions. 16–20 h before harvest, cells were labeled with 1 μCi/well [3H]thymidine (ICN, Costa Mesa, CA). Cells were harvested using an Inotech harvester (Inotech, Lansing, MI) and counted in a 2500 TR Packard liquid scintillation counter (Packard Instruments Co.).

For cell cycle analysis, appropriately treated cells were washed in cold phosphate-buffered saline, treated with RNase (0.5 mg/ml), and stained with 40 μg/ml propidium iodide. Samples were analyzed by flow cytometry in a linear scale using a FACScan cytometer (Becton Dickinson, Mountain View, CA). Fluorescence data were analyzed by the CELL Quest software (Becton Dickinson).

Receptor Activation on the (TREpal)2—The selectivity of the different retinoids was studied in transfection experiments as described previously (22). Briefly, CV-1 cells were cotransfected with the (TREpal)2-tk-CAT construct and each of the retinoid receptor expression vectors. After treatment with the different retinoids, CAT activity was analyzed.

RNA Preparation and Northern Blot Analysis—Following treatment, cells were detached from the dish by mild trypsin digestion, washed twice with ice-cold phosphate-buffered saline, and processed for RNA extraction with the RNAeasy kit (Quiagen Inc., Chatsworth, CA) according to the manufacturer’s instructions. Total RNA (15 μg) was electrophoresed on a denaturing formaldehyde-agarose gel and transferred to nylon Gene Screen Plus hybridization membranes (DuPont) by overnight blotting. Blots were hybridized overnight with 3 × 106 cpm of 32P-labeled DNA probes/ml. DNA probes were labeled by random priming to an efficiency of 0.5–1 × 106 cpm/μg. Filters were washed in a solution containing 0.3 M sodium chloride, 0.03 M sodium citrate dehy-
drate, and 0.5% sodium dodecyl sulfate at 65 °C and autoradiographed at ~70 °C with intensifying screens.

RT-PCR Analysis of RXRα Expression—DNase-treated total RNA (0.5 μg) was subjected to reverse transcription reaction using Moloney murine leukemia virus reverse transcriptase kit according to the manufacturer’s instructions (Life Technologies, Inc.) in a final volume of 20 μl for 1 h at 37 °C. An aliquot (2 μl) of the reverse transcription reaction was subjected to 30 PCR cycles (1 min at 94 °C, 1 min at 68 °C, 1 min at 72 °C) in 1 × PCR buffer (Perkin-Elmer), 50 pmol of each primer, 1.5 mM MgCl2, 200 μM dATP, dCTP, dGTP, dTTP, 2.5 units of AmpliTaq polymerase (Perkin-Elmer). Specific primers (P1, 5′-TTCTCTGCTT-GCCCTGTGGT-3′; PR2, 5′-TCAGCTATGTCAGGTCAG-3′) were used to amplify a 154-base pair fragment from nucleotide 1554 to 1707 of the human RXRα sequence (40). In the same PCR reaction, the ribosomal protein S12 cDNA was amplified as an internal control by means of specific primers (A, 5′-GAAGGCCATGCTGCTGCTG-3′; B, 5′-CTCTAACATACCATCCTGG-3′).

RESULTS

Expression of Retinoid Receptors in KCNR NB Cell Line—To study which of the RAR subtypes contribute to the response of KCNR NB cells to retinoids, we first sought to analyze the expression of RARα, RARβ, and RARγ by Northern blot in control and ATRA- or 9CRA-treated cells. A readily detectable level of the 3.9- and 2.9-kilobase RARα transcripts and the 3.5- and 3.2-kilobase RARγ transcripts were constitutively expressed in KCNR cells (Fig. 1A). The 3.4- and 3.0-kilobase RARβ mRNAs were detected only after prolonged exposure of the same blot (not shown). Both ATRA and 9CRA induced a 2- to 3-fold increase in the steady state level of RARα and RARγ mRNAs, whereas RARβ mRNA was induced >30-fold compared with solvent-treated cells. RXRa mRNA expression was below the level of detection of our Northern blot analysis. Therefore RT-PCR was used to analyze its expression in KCNR cells (Fig. 1B). We observed no major change in its expression after treatment of cells with either ATRA or 9CRA by an ordinary (Fig. 1B) or semiquan-
titative (not shown) RT-PCR.

Effect of the Retinoids on the Morphological Differentiation of Neuroblastoma Cells—KCNR cells maintained in a medium supplemented with charcoal-stripped serum grow in aggregates of slightly fused or rounded cells, extending very rare and short neurites (Fig. 2A). Exposure to either ATRA or 9CRA (10−6 M) induces morphological changes starting with cell flattening and disaggregation and culminating with the extension of neurites exceeding at least twice the length of the cell soma. Extension of neurites occurs in most of the cell population after 4 days of treatment (Fig. 2B), and >90% of the cells extend neurites by 7–8 days (not shown).
To determine which of the retinoid receptor classes and subtypes are involved in transducing the signal(s) leading to such a morphological response to ATRA or 9CRA, various retinoid analogs were tested on KCNR cells in doses ranging from $10^{-2}$ to $5 \times 10^{-6} \text{M}$. The selectivity of these SRI retinoids was previously established on the (TREpal)$_2$ RARE, and the results are summarized in Table I. Am580 is a RAR$_a$-selective ligand extensively characterized (30, 32, 41, 42). Treatment of the cells with SR11383 entirely reproduced the pattern of events observed after treatment with ATRA or 9CRA. In particular, cell flattening and disaggregation were observed after 4 days of treatment at as low as $10^{-6} \text{M}$ (not shown), a concentration at which this ligand selectively activates the (TREpal)$_2$ through RAR$_b$ (Table I). The extension of relatively long neurites on the majority of the cells was also readily detectable at $10^{-6} \text{M}$ (not shown), and the maximum, at $10^{-7} \text{M}$ (Fig. 2C). However, at the latter concentration, SR11383 showed 58% of the activity of ATRA on the (TREpal)$_2$ through RAR$_b$ and 31% through RAR$_a$ (Table I). Similar morphological changes were also observed upon treatment of the cells with $10^{-8} \text{M}$ (not shown) or $10^{-7} \text{M}$ Am580 (Fig. 2D), which at this concentration predominantly activated the (TREpal)$_2$ through RAR$_a$ (30, 32, 41, 42). In contrast, SR11254 only induced cell flattening without a marked effect on cell spreading and neurite extension even at a concentration as high as $10^{-6} \text{M}$ (Fig. 2G). At this concentration SR11254 showed 84% of the activity of ATRA on the (TREpal)$_2$ through RAR$_a$ and 46% through RAR$_b$ (Table I). No morphological changes were observed in response to the RXR-selective

![Fig. 2. Effect of the retinoids on the morphological differentiation of KCNR cells.](image-url)
ligands SR11246 (Fig. 2I) and SR11234 (not shown) at similar concentrations. However, both SR11246 (Fig. 2, E, F, and H) and SR11234 (not shown) increased the morphologic response to Am580 (Fig. 2E), SR11383 (Fig. 2F), and SR11254 (Fig. 2H), suggesting that ligand-induced activation of RXR cooperates with liganded RARα, RARβ, and RARγ to induce morphological differentiation in KCNR NB cells.

Effect of the Retinoids on the Proliferation of Neuroblastoma Cells—ATRA induces a dose-dependent growth inhibition in NB cells (43), which can be monitored by the decrease in [3H]Thy uptake. In the KCNR cell line, doses of ATRA ranging between $10^{-7}$ and $5 \times 10^{-6}$ M induced a 30–70% reduction in [3H]Thy uptake (Fig. 3A). Similar results were observed upon treatment with 9CRA, although its maximum effect was detected at $1 \times 10^{-6}$ M (Fig. 3A). To investigate the contribution of the different retinoid receptors to the growth inhibitory response of KCNR cells to natural retinoids, we tested the effects of the above-mentioned selective compounds on thymidine incorporation experiments. Only SR11383 was able to induce growth inhibition as efficiently as ATRA or 9CRA, show-

**TABLE I**

Receptor selectivity of the different retinoids on the (TREpal)2

| Retinoids     | RARα | RARβ | RARγ | RXRα |
|--------------|------|------|------|------|
| AC50 log M   |      |      |      |      |
| SR11383      | -2   | 22   | 66   | 71   |
| SR11254      | 7    | 7    | 8    | 13   |
| SR11246      | -1   | -3   | 2    | 6    |
| SR11234      | 8    | 0    | 0    | 0    |
| SR11286      | ND   | 6    | 42   | 80   |
| SR11288      | -2   | 1    | 5    | 42   |
| SR11381      | -3   | -1   | 4    | 29   |

* For each concentration (log M) of the retinoid being used, results are expressed as percent activation compared with activation by 1 µM ATRA as 100% for the RARs and 1 µM 9CRA as 100% for RXRα.

**AC50** indicates the concentration of each retinoid required to give 50% of the response of 1 µM ATRA for the RARs and 1 µM 9CRA for RXRα.

**ND**, not determined.

**FIG. 3. Effect of the retinoids on the proliferation of KCNR cells.** Quadruplicate cultures of KCNR cells plated at $10^4$ cells/well were grown for 4 days in medium containing charcoal-stripped FBS in the presence of either solvent or the different retinoids alone or in combination in 96-well plates. After an overnight pulse with [3H]Thy, cells were harvested and counted for incorporated radioactivity. For each retinoid, results are expressed as percentage of [3H]Thy incorporation compared with solvent-treated cells. Data represent mean ± S.D. of quadruplicate cultures. The different retinoids were applied at concentrations ranging from $10^{-2}$ to $5 \times 10^{-6}$ M (A), $10^{-2}$ M (B and C), and $10^{-6}$ M (D). These results are representative of at least three separate experiments.
ing a 35 and 70% reduction in \[^{3}H\]Thy uptake when applied at concentrations of 10\(^{-7}\) and 5 \times 10\(^{-6}\) M, respectively (Fig. 3A). Within this concentration range, SR11383 activates the (TRE-pal)\(_2\) through both RAR\(\beta\) and RAR\(\alpha\) (Table I). Am580 decreased \[^{3}H\]Thy uptake only by 20–25% between 1 \times 10\(^{-6}\) and 5 \times 10\(^{-6}\) M (Fig. 3A), which was confirmed in four independent experiments but was ineffective at lower and more selective concentrations. No growth inhibition occurred in cells treated with SR11254, SR11246 (Fig. 3A), or SR11234 (not shown), even at micromolar concentrations. Since we observed a cooperative effect of RXR-selective ligands with RAR-selective ligands in inducing morphological differentiation of KCNR cells, we investigated the effect of these combinations on cell proliferation. A suboptimal concentration (10\(^{-7}\) M) of SR11383 reduced \[^{3}H\]Thy incorporation to 65% that of the control (Fig. 3, A and B). A further reduction to 20 or 40% was obtained by adding 10\(^{-7}\) M RXR-selective ligand SR11246 or SR11234, respectively, but not by adding Am580 (Fig. 3B). A more selective concentration (10\(^{-8}\) M) of SR11383 was almost inactive alone, but in combination with 10\(^{-8}\) M SR11246 or SR11234 reduced \[^{3}H\]Thy uptake by >40% (not shown). At this concentration, SR11383 activates the (TRE-pal)\(_2\) mostly (61%) through RAR\(\beta\) and only to a lesser extent (21%) through RAR\(\alpha\) (Table I). These observations in KCNR cells and the identical responses we observed in the LAN-5 NB cell line\(^2\) suggest that RAR\(\beta\) mediates growth inhibitory signals in response to retinoids in these and possibly other NB cells.

To further study the contribution of RAR\(\beta\) to NB cell growth inhibition by natural retinoids, we tested three additional synthetic analogs which, although not highly selective, preferentially activate RAR\(\beta\) in transactivation experiments (Table I). At 10\(^{-6}\) M, the RAR/RXR panagonists SR11288 and SR11286 induced a 60 and 40% inhibition of \[^{3}H\]Thy uptake in KCNR cells, respectively. RAR class-selective SR11381 was inactive, even between 1 \times 10\(^{-6}\) (Fig. 4A) and 5 \times 10\(^{-6}\) M (not shown). Addition of RXR-selective SR11246 did not potentiate the growth inhibitory properties of the panagonist SR11288 (Fig. 4A), which at this concentration strongly activates transcrip-

tion of the (TRE-pal)\(_2\) through RAR\(\beta\) (98%), RXR\(\alpha\) (84%), and to a far less extent, through RAR\(\alpha\) (42%) (Table I). SR11246 did increase to 60% the growth inhibition caused by SR11286 (Fig. 4A), which is a less potent RXR\(\alpha\) activator (56%) but a very strong RAR\(\beta\) activator (232%) on the (TRE-pal)\(_2\) (Table I). Moreover, in combination with SR11246, the otherwise inactive SR11381 inhibited \[^{3}H\]Thy uptake to the same extent as did SR11286 (Fig. 4A).

At 10\(^{-7}\) M, Am580 was inactive in inhibiting proliferation. However, the addition of 10\(^{-7}\) M of either of the two RXR-selective SR11246 or SR11234 to 10\(^{-7}\) M Am580 caused a 60–70% reduction in \[^{3}H\]Thy incorporation (Fig. 3C). This effect was not merely dependent on the combination of two retinoids, since the combination of 10\(^{-7}\) or 10\(^{-6}\) M SR11254 with 10\(^{-7}\) or 10\(^{-6}\) M Am580 failed to produce any additive or cooperative effect on cell proliferation (Fig. 3, C and D). SR11254 alone at 10\(^{-6}\) M had no effect at all, but the addition of 10\(^{-6}\) M of either of the RXR-selective SR11246 or SR11234 decreased \[^{3}H\]Thy uptake by 50% (Fig. 3D). Simultaneous administration of the two RXR ligands up to 10\(^{-6}\) M did not influence the growth of KCNR cells (not shown).

In a number of cancer cell lines, growth inhibition by retinoids was reported to be associated with inhibition of AP-1 transcriptional activity (16, 17). To investigate the contribution of this pathway to the antiproliferative response that retinoids induce in NB cells, we also analyzed incorporation of \[^{3}H\]Thy by KCNR cells in the presence of the dissociated retinoids, SR11302 and SR11238. These retinoids fail to activate gene expression through retinoid receptors but were reported to antagonize AP-1-dependent transcription (16). Either alone or in combination with SR11246, SR11302 and SR11238 did not affect KCNR cell proliferation even at concentrations as high as 1 \times 10\(^{-6}\) M (Fig. 4B) or 5 \times 10\(^{-6}\) M (not shown), suggesting that NB cell antiproliferative response to retinoids does not depend upon AP-1 antagonism. This observation is also in agreement with the lack of sensitivity of KCNR cells to the phorbol ester 12-myristate 13-acetate.\(^3\)

**Growth Inhibition of KCNR Cells by the Different Retinoids Is Due to a Cell Cycle Arrest**—The decrease in thymidine incorporation induced in KCNR cells by ATRA or 9CRA is a consequence of the arrest of the majority of cells in the G\(_1\) phase of the cell cycle, with concomitant reduction in the number of cells entering S phase, as demonstrated by FACS analysis (33) (Fig. 5 A–C). The decreased \[^{3}H\]Thy incorporation observed in KCNR cells upon treatment with the selective retinoids could be dependent on a similar effect on the cell cycle. However, it could also be due to an increased cell death or to a reduced doubling time of the culture. To verify which of these hypotheses mostly accounted for the reduction in \[^{3}H\]Thy incorporation, we performed FACS analysis on cells treated for 4 days with the different ligands or their combinations. Similar to ATRA, 10\(^{-7}\) M SR11383 induced a reduction in the number of cells entering the S phase and an accumulation of cells in G\(_1\) (Fig. 5D). This effect was enhanced by the addition of the same concentration of SR11246 or SR11234, with up to 79% of the cells arrested in G\(_1\) and only 4.4% of the cells left in S phase (not shown). As expected from the absence of response in the \[^{3}H\]Thy uptake assay, cells treated with 10\(^{-7}\) M Am580 (Fig. 5E) or with 10\(^{-6}\) M SR11254 (Fig. 5F) did not substantially differ in their cell cycle distribution from solvent-treated cells (Fig. 5A). However, combination of SR11246 (Fig. 5, F and I) or SR11234 (not shown) with either Am580 or SR11254 also resulted in an efficient cell cycle arrest in G\(_1\). None of the treatment caused either visible cell death in the culture or accumu-

\(^2\) G. Gianninni, unpublished observation.

\(^3\) C. J. Thiele, unpublished observation.
lation of cells with a $< 2N$ DNA content (Fig. 5), suggesting that no apoptosis was induced by any of the ligands or ligand combinations within the examined time frame. On the contrary, these data indicated that the decrease in $[3H]$Thy incorporation induced by the effective associations of retinoid analogs was due to the withdrawal of KCNR cells from the cell cycle.

Effect of the Retinoids on Gene Expression—In NB cells, RA regulates the expression of a number of genes. Among them the down-regulation of N-myc and the up-regulation of TrkB and RAR$\beta$ were shown to be temporally and causally linked to the control of cell growth and/or differentiation (33–35, 37). In addition, RAR$\beta$ (44), N-myc (45, 46), and TrkB (36) were shown to be regulated by RA at the transcriptional level. To determine whether specific receptor-selective retinoids can differentially regulate genes whose expression is associated with differentiation and/or growth inhibition, the steady state levels of RAR$\beta$, N-myc, and TrkB were assayed by Northern analysis on total RNA extracted from KCNR cells treated with the different retinoids alone or in combination. In addition to the observed effects on the morphological differentiation and proliferation, treatment of KCNR cells with $10^{-7} M$ SR11383 induced an increase in the steady state levels of TrkB and RAR$\beta$ mRNAs (Fig. 6A, lane 4), which corresponded to 84 and 74% of the effect of the optimal concentration ($10^{-6} M$) of ATRA on the same genes (Fig. 6B). It also greatly reduced the N-myc mRNA steady state level compared with solvent-treated KCNR cells (Fig. 6A, lane 4) to 81% of the reduction caused by $10^{-6} M$ ATRA (Fig. 6B). The activity of $10^{-7} M$ SR11383 on the regulation of the expression of TrkB, N-myc, or RAR$\beta$ was enhanced dramatically (130–140% that of the effect of ATRA) by $10^{-7} M$ RXR-selective SR11246 and to a lower extent by the less potent RXR-selective SR11234 (Fig. 6A, lanes 7 and 12, and B).

At $10^{-7} M$, Am580 induced the morphological differentiation but not the growth inhibition of KCNR cells. At this concentration, it efficiently increased the steady state levels of TrkB and RAR$\beta$ mRNAs (Fig. 6A, lane 5) to 84 and 56%, respectively, of those found using optimal concentration ($10^{-6} M$) of ATRA (Fig. 6B). However, it very inefficiently decreased the N-myc steady
state level (Fig. 6A, lane 5), showing only 22% that of the activity of 10^{-6} M ATRA (Fig. 6B). The induction of TrkB or RARβ by 10^{-7} M Am580 was enhanced markedly to 120–140% of ATRA effect by the addition of 10^{-7} M RXR-selective SR11246 and to a lower extent by the less active RXR-selective SR11234 (Fig. 6A, lanes 8 and 13, and B). These combinations also decreased N-myc steady state levels, with an effect equal to 65% that of 10^{-6} M ATRA (Fig. 6B).

Between 10^{-7} and 10^{-6} M SR11254 (Fig. 6A, lane 9), SR11246 (Fig. 6A, lane 6), and SR11234 (Fig. 6A, lane 11) did not significantly affect TrkB, N-myc, and RARβ mRNA expression. However, the combination of 10^{-6} M SR11254 and SR11246 increased the steady state levels of TrkB and RARβ mRNAs (Fig. 6A, lane 10) to 58 and 36% that induced by ATRA, respectively (Fig. 6B), and decreased N-myc expression (Fig. 6A, lane 10) to 40% of ATRA activity (Fig. 6B). In combination with the less potent SR11234, 10^{-6} M SR11254 produced qualitatively similar results (Fig. 6A, lane 14 and B).

DISCUSSION

KCNR cells represent a prototype for a number of so-called neuroblast-like (or N-type) neuroblastoma cell lines that undergo morphological differentiation and growth arrest upon treatment with RA. Although it was previously suggested that ATRA and 9CRA may elicit slightly different responses on NB cells (44, 47), the activities of these two compounds were very similar in KCNR cells. Like other NB cell lines (39), KCNR cells express a complex pattern of retinoid receptor isoforms that may potentially contribute to their responses to retinoids. We have analyzed the effects of a panel of receptor-selective retinoids on the growth, morphological differentiation, and gene expression of KCNR cells to gain a better understanding of which retinoid receptors contribute to these responses in NB cells. The results presented in this report are not restricted to the KCNR cell line, as they were also reproduced in the LAN-5 NB cell line.2

Ligand-dependent Activation of RXRα Potentiates the Activity of RARs in NB Cells—Although RXR-selective ligands are thought to promote transcription through the activation of RXR/RXR homodimers (18), neither SR11246 nor SR11234 alone were able to elicit morphological differentiation, growth arrest, and gene expression in KCNR cells, suggesting that RXR/RXR homodimers and the genes they may regulate do not contribute to the biological responses of NB cells to natural retinoids. Among the RAR-selective retinoids, SR11383 induced both growth inhibition and differentiation, whereas Am580 only induced differentiation (discussed below) when used alone. SR11254 and SR11381 were almost inactive. However, addition of the RXR-selective ligand SR11246 (and SR11234 when tested) potentiated the action of all the RAR ligands on proliferation, differentiation, and gene expression experiments, particularly when suboptimal doses of the RAR-selective retinoids were used. The enhancement of the activity of RAR-selective ligands due to the addition of RXR-selective ligands suggests that activation of the RXR within the RXR/RAR heterodimer cooperatively contributes to the differentiation, growth inhibition, and gene expression induced by retinoids in NB cells. These findings are in agreement with recent literature which indicates that, although ligand binding to the RAR in the RXR/RAR heterodimer is required and sufficient for both DNA binding and transcriptional activation of several responsive elements (12, 19–21), ligand binding to the RXR potentiates the transcriptional activity of RXR/RAR heterodimers (11, 31, 41, 48).

Three Receptor Heterodimers Mediate Responsiveness of NB Cells to Retinoids—Previous studies have suggested that specific heterodimers may mediate responses to retinoids in different cell culture models. RXR/RARγ heterodimers have been proposed to transduce signals in response to retinoids in embryonal carcinoma cells. In fact, RARγ-null F9 cells are less sensitive (28), and RXR-null F9 cells are completely resistant (49) to retinoids. In addition, overexpression of RARα but not RARβ can fully reconstitute the response to retinoids in the RARγ-null F9 cells (24, 29). Use of selective retinoids has revealed that RARα plays a major role in the differentiation response of NB4 and HL60 leukemia cell lines (30–32, 50). One limitation of receptor-selective ligands is that little is known of the potential for variability in the uptake and/or the intracellular metabolism of the different retinoids in CV-1 cells, the
recipient for the transcriptional studies, compared with tumor cells. However, if there were gross differences in metabolism among different cell types, one might anticipate differences in the results obtained using agonists and those using other methods such as gene knockouts. In fact, RARγ has been implicated by both agonist and gene knockout studies to be critical in mediating retinoid differentiation in F9 cells (29). Furthermore, data generated by the use of an antisense RARβ expression construct, a selective agonist, and a selective antagonist all pointed to a role of RARβ in mediating the growth inhibition of hormone-dependent breast cancer cells (51). Clearly, methods in addition to the use of agonists are needed to unequivocally define the receptors involved in mediating these complex biological processes.

RARα is constitutively expressed in KCNR cells. Its role in mediating the action of retinoids in NB cells is indicated by the effects of Am580. This retinoid is generally considered RARα-selective if used at nanomolar concentrations (30, 32, 41, 42). Up to $10^{-7}$ M Am580 did not affect the growth of NB cells, yet it did induce morphological differentiation. These results are consistent with the observation that it efficiently induced TrkB mRNA, whose expression was previously shown to be associated with neuritogenesis and differentiation in NB cells (37). In contrast, Am580 did not efficiently induce the down-regulation of N-myc mRNA that is associated with and required for the growth inhibitory responses of NB cells to retinoids (33, 34). These results suggest that perhaps the selective activation of RARα by Am580 alone may separate the neurotrophic pathway from the growth inhibitory one. A similar dissociation of the growth and differentiation pathways was also observed when low concentrations ($10^{-9}$ M) of ATRA were found to induce neurite extension and TrkB but not inhibit the growth of KCNR or SY5Y NB cells (37, 43). In combination with the RXRα-selective ligand SR11246, $10^{-7}$ M Am580 induced growth inhibition, differentiation, and expression of TrkB and RARβ mRNAs even more efficiently than ATRA. Under these conditions, it also induced 5-fold N-myc mRNA expression (with an activity comparable to 65% of the effect of $10^{-6}$ M ATRA). Since TrkB, RARβ, and N-myc mRNA expression is regulated by retinoids at the transcriptional level (36, 44–46), the less efficient regulation of N-myc compared with the effect on TrkB and RARβ expression may reflect a differential capability of Am580 to regulate N-myc versus TrkB or RARβ promoters. This may depend on the nature of the yet uncharactertized N-myc retinoic responsive element in contrast to the canonical RARE, which regulates RARβ (52, 53), or the putative RARE in the TrkB promoter. However, it is also possible that the individual ligands have different effects on the expression of selected genes, since we have recently identified mRNAs by differential display whose expression is selectively affected by specific ligand combinations.2

The role of RARβ in mediating responses to retinoids in NB cells was tested by means of several ligands. SR11383, SR11286, SR11288, and SR11381 displayed a preferential activation of the (TREpalm)2 through RARβ. All these ligands were potently effective in mediating growth arrest and differentiation of KCNR cells either alone (SR11383, SR11286, and SR11288) and/or in combination with an RXRα-selective ligand (SR11383, SR11286, SR11288, and SR11381). This indicates that RARβ activation is relevant for the induction of growth inhibition and differentiation of NB cells. However, the effectiveness of these retinoids on the growth inhibition assay did not overlap their effectiveness in the transcriptional assay. It has been shown that, in addition to their affinity for a given receptor subtype, the activity of specific retinoids may also be strongly dependent on the specific RARE being activated (12). Therefore, the apparent discrepancy between the effects of some retinoids on the transcriptional activation of the (TREpalm)2 in the presence of an overexpressed RAR subtype versus the induction of complex biological responses evaluated in cells containing physiologic levels of receptors may depend on a different sensitivity of the specific regulatory elements involved in the control of the genes that mediate these biologic pathways.

SR11383 is an exceptionally active retinoid. At $10^{-7}$ M, it was an efficient regulator of TrkB, RARβ, and N-myc gene expression and, in the presence of an RXR-selective ligand, it was far more effective than $10^{-6}$ M ATRA. In addition, in the presence of an RXRα-selective ligand, SR11383 efficiently regulated growth inhibition and differentiation of KCNR and LAN-5 cells at doses as low as $10^{-8}$ M (not shown). At this concentration, SR11383 appeared to be rather RARβ selective for the activation of the (TREpalm)2, which reinforces the idea that RARβ may play an important role in mediating responses to RA in NB cells. This conclusion is also indicated by the recent observation that RARβ overexpression in BE(2)-C NB cells, but not RXRα and RARγ overexpression, causes growth inhibition and enhanced sensitivity to retinoids (38).

SR11254 is a good activator of the (TREpalm)2 element, with a preferential selectivity for RARγ. Although basically inactive if used alone, SR11254 also induced a moderate effect on cell growth, morphological differentiation, and regulation of gene expression in the presence of the RXRα-selective ligand SR11246. Higher concentrations ($10^{-6}$ M) of SR11254 and SR11246 were needed to inhibit proliferation, stimulate differentiation, and control gene expression in KCNR cells. At this concentration, SR11254 also activated RARβ in the transcriptional assays. However, the effect on growth does not appear to be dependent on RARβ activation because even in the presence of an RXRα-selective ligand, SR11254 failed to inhibit growth of HL-60 cells,2 which lack detectable expression of RARγ (54). If SR11254 were activating via RARβ, it should be active in HL-60, as both RARα (SR11383) or RARα (Am580) agonists induce differentiation in HL-60.2 Therefore we suggest that the inhibitory effect of SR11254 observed in KCNR and LAN-5 cells was not due to its interaction with RARα or RARβ but rather RARγ. That RARγ may have a role in mediating retinoid effects in NB cells is also supported by the work of Marshall and co-workers (38), who have shown that overexpression of RARγ in BE(2)-C cells may inhibit tumorigenicity (55) and that CD666, a RARγ agonist, inhibits cell growth.

In conclusion, our study indicates that conformationally restricted retinoids regulate proliferation and differentiation of neuroblastoma cells to the same extent as naturally occurring retinoids through the activation of three distinct receptor heterodimers: RXRα/RARα, RXRα/RARβ, and RXRα/RARγ. In addition, the growth inhibitory response activated by retinoids in NB cells did not involve the AP-1 antagonistic effect known to occur in other tumor cell lines (16). This was demonstrated by the lack of activity of the dissociated ligands SR11302 and SR11238. These ligands do not transactivate reporter genes but can still antagonize AP-1-dependent transcription (16).

Retinoids are successfully employed in clinical protocols for a number of diseases, including blood and neuroectodermal malignancies and skin disorders. Phase I clinical trials using 9CRA and phase II trials using ATRA in combination with interferon α are currently being used in treating neuroblas-

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4 Y. Kitajima, K. Matsumoto, M. Y. Morrill, C. Gaetano, and C. J. Thiele, submitted for publication.
We have shown here that particular combinations of synthetic retinoids like SR1183/9R11246 can inhibit NB cell proliferation more efficiently than naturally occurring retinoids under the same conditions. We expect that the development of synthetic ligands that alone or in combination can be applied at lower and possibly less toxic concentrations may improve the therapeutic utility of retinoids.

Acknowledgments—We thank Dr. Peter D. Hobbs, Dr. Ling Jong, and Dr. Guo-guan Chen for their valuable work in the synthesis and characterization of the transcriptional activity of some of the retinoids. We are grateful to Dr. Saverio Minucci and Dr. Jim Stauffer for the critical reading of the manuscript and to Dr. L. Gudas for helpful discussions.

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