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A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects

(retinoids/cell differentiation/HL-60/B lymphocytes)

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ABSTRACT **Retinoic acid (RA) exerts its pleiotropic effects on cell growth and differentiation through the activation of a family of transcription factors—the RA receptors (RARs). Three subtypes of these receptors exist, RARα, RARβ, and RARγ. The receptors are differentially expressed in different cell types and stages of development, suggesting that they may regulate different sets of genes. We have identified a synthetic retinoid with the characteristics of a selective RARα antagonist. This antagonist counteracts RA effects on HL-60 cell differentiation and on B-lymphocyte polyclonal activation. Beyond its potential practical relevance, this and other specific antagonists will be useful to dissect the RAR system and to assign to one given receptor each of the many RA-regulated functions.**

The natural retinol (vitamin A) derivative retinoic acid (RA) is known to have profound effects on cell growth and differentiation (1) and to be essential for normal embryonic development (2). While RA and some synthetic analogs (retinoids) are useful in the control of some tumors (3) as well as of nonmalignant hyperproliferative conditions of the skin (4), they are, at high concentrations, teratogenic (5).

The pleiotropic effects of retinoids are mediated by two known families of nuclear receptors, both belonging to the steroid–thyroid hormone receptor superfamily of ligand-inducible transcriptional regulators (6, 7). The RA receptor (RAR) gene family comprises three subtypes—RARα (8, 9), RARβ (10–12), and RARγ (13, 14)—with each gene encoding a variable number of isoforms arising by differential splicing of two primary RNA transcripts (15–17). All receptors of the RAR family bind RA with comparable affinity (18). The retinoid receptors of the second family (RXR) do not bind the major form of RA (all-*trans*-RA) (19). They bind instead the 9-*cis* stereoisomer of RA (20, 21).

Transcription of some RAR genes themselves is RA sensitive (22–25). Also, the expression of some of the cellular retinol- or RA-binding proteins (CRBP and CRABP), putatively involved in the storage, transport, and/or metabolism of retinol and RA, is differentially regulated by RA in a receptor-specific manner (26–28). The RA-related molecules represent, therefore, an autoregulated system.

RAR types and isoforms, as well as RXRα and RXRβ, are differentially expressed both spatially and temporally (15–18, 29–32). They might therefore regulate different target genes during embryonic and adult life, as well as in specific cell types at different stages of differentiation. RARα is the most ubiquitously expressed, while RARβ and RARγ display a more restricted pattern of distribution, with RARγ being predominantly expressed in the skin (31).

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It seems reasonable to assume that the multiple effects of RA could be dissociated by specific ligands for each of the known receptors, and/or by receptor-specific antagonists, so as to obtain the desired beneficial effects while limiting the unwanted side effects. Retinoids with a good degree of selectivity have been described (33), and we have ourselves obtained retinoids with strong preference for each of the RAR subtypes (ref. 34; unpublished results). We have also found retinoids with selective binding to RARα that are not competent to activate the receptor in a transactivation assay. We describe here one such retinoid, which prevents RARα activation by RA, thus displaying the characteristics of a RARα-selective antagonist. The retinoid counteracts RA effects in some but not other functional systems. The relevance of this and similar molecules for restricting the spectrum of RA effects and for assigning to a specific receptor one given RA-regulated function is discussed.

MATERIALS AND METHODS

Retinoids. All-*trans*-RA and analogues were synthetized at Hoffmann–La Roche. All-*trans*-[^{3}H]RA (50 Ci/mmol; 1 Ci = 37 GBq) was obtained from DuPont/NEN. Retinoids were solubilized in dimethyl sulfoxide (DMSO) as 10 mM stock solutions and kept at −80°C. Further dilutions were made in phosphate-buffered saline containing 1% gelatin and 4% DMSO or in the appropriate culture medium. Some retinoids were unstable upon storage and new solutions were prepared for each experiment.

Plasmids. The plasmid pCH110 (35) was from Pharmacia. Chimeric RAR cDNAs (RARα-ER.CAS or RARβ-ER.CAS) were provided by P. Chambon (Faculté de Médecine, Strasbourg, France) (9, 11). They contain the DNA-binding region of the estrogen receptor (ER) [amino acids (aa) 185–250] replacing the DNA-binding region of RARα (aa 88–153) or RARβ (aa 81–146). For construction of the corresponding RARγ chimeric receptor, the mouse RARγ cDNA was cloned by PCR from mouse F9 cells, and the correctness of the sequence was confirmed. The clone was then altered by site-directed mutagenesis to encode the human RARγ protein. The ER DNA-binding domain was cloned by PCR from human placental cDNA. The DNA-binding domain of the RARγ receptor (aa 90–155) was replaced by the ER DNA-binding domain (aa 185–250) to form the chimeric receptor. These chimeric receptors then recognize the estrogen response element. Through the use of chimeric receptors in transcription activation assays, any background induction due to endogenous RARs is avoided. As a reporter system,

Abbreviations: RA, retinoic acid; RAR, RA receptor; SeAP, secreted alkaline phosphatase; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; aa, amino acids; ER, estrogen receptor; LPS, lipopolysaccharide.

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we used the SeAP (secreted alkaline phosphatase) gene (36) under control of the vitellogenin estrogen response element fused to the herpes simplex thymidine kinase promoter (vit-TK-SeAP, pPL141).

For expression of the ligand-binding domain (DEF) of RARα (the C-terminal 262 aa) and RARβ (the C-terminal 255 aa) in *Escherichia coli*, the expression vector pDS56/RBSII was used (37, 38). For expression of RARγ-DEF (the C-terminal 299 aa), the cDNA was cloned by PCR into the T7 *E. coli* expression vector pET-3a (39). RARα-DEF and RARβ-DEF were expressed in *E. coli* M-15 (37); RARγ-DEF was expressed in *E. coli* BL21(DE3)pLysS (39) after isopropyl β-D-thiogalactopyranoside induction to a level of 5–10% of the total *E. coli* protein (as estimated by SDS/PAGE). Receptors were solubilized from the cells by lysozyme treatment (2.8 mg/ml) in 25 mM Tris·HCl, pH 8.0/10 mM EDTA/50 mM glucose/2 mM dithiothreitol for 10 min at room temperature. The digest was homogenized after the addition of 0.3 M NaCl/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride (final concentrations) in a Polytron at 4°C (maximal speed for 2 min). Aggregates were centrifuged at 10,000 × *g* for 15 min. The supernatants, referred to as crude receptor extracts, were stored at −80°C.

Retinoid-Binding Assay. The DEF domains of the RARs were used to measure retinoid binding since this domain was found to have the same binding characteristics as the full-length receptors (ref. 38; unpublished results). In routine assays, 0.2–0.4 pmol of receptors (DEF domain, crude extract) were incubated in glass microtubes in 0.2 ml of 50 mM Tris·HCl, pH 7.2/50 mM NaCl/2 mM EDTA (binding buffer) containing 0.5% gelatin, 1% DMSO, trypsin inhibitor (1 μg/ml), aprotinin (100 trypsin inhibitor units per ml), 2–5 nM [^{3}H]RA, and various concentrations of unlabeled retinoids. After 3 hr of incubation at room temperature, 0.15 ml of chilled charcoal/dextran suspension (5% Norit A/0.5% dextran in binding buffer) was added for 15 min at 4°C. The tubes were centrifuged at 12,000 × *g* for 10 min and the supernatants were subjected to liquid scintillation counting. Retinoid-binding assays were performed under equilibrium conditions. Binding parameters were calculated from competition curves using the EQUILIBRIUM BINDING DATA ANALYSIS computer program of Biosoft.

Transient Transfection and SeAP Assay. COS-1 cells were grown adherent to plastic dishes in DMEM (GIBCO) supplemented with 10% fetal calf serum (FCS) (GIBCO). For transient transfection, cells were replated at 80% confluence in 10-cm dishes 24 hr before the experiment. Transfection was performed by the DEAE-dextran method (40), using 4–20 μg of total plasmid DNA (SeAP reporter/different RAR-ER expression vectors/pCH110, 5:1:1) per dish in DMEM plus 10% charcoal-stripped FCS. The β-galactosidase expression vector pCH110 served to correct for variation in transfection efficiency. The β-galactosidase expression itself was slightly depressed (<10%) at high doses of retinoid agonist or antagonist, but this factor has no significant influence on our overall results.

Eighteen hours later, the cells were trypsin treated and replated at 80–90% confluence in 96-well plates in DMEM (lacking phenol red) plus 10% charcoal-stripped FCS. After 4 hr, various concentrations of retinoids were added to triplicate wells. At the end of the incubation (36–48 hr) the cell supernatants were assayed for SeAP activity. After a 5-min preincubation at 65°C to inactivate nonspecific phosphatases, 2 μl of supernatant was combined with 100 μl of substrate (Lumi-Phos 530, Lumigen, Detroit) and incubated for 1 hr at 37°C. For determination of β-galactosidase activity, the medium was aspirated and 100 μl of β-galactosidase substrate was added (Lumi-Gal 530, Lumigen) for 1 hr at 37°C. The luminescent reaction products were measured in a 96-well luminometer (Luminoscan, Flow Laboratories). The data are represented as relative SeAP activity, defined as SeAP activity divided by β-galactosidase activity. Absolute SeAP activity measurements were in the range of 2–80 milliunits (1 milliunit is the amount of alkaline phosphatase that will hydrolyze 1.0 pmol of *p*-nitrophenylphosphate per min) (36). Triplicate values varied by 5–10%.

HL-60 Cultures. The human promyelocytic cell line HL-60 was maintained in culture in RPMI 1640 medium (Seromed, Munich) supplemented with glutamine, sodium pyruvate, antibiotics, and 10% FCS (Boehringer Mannheim). Cultures were exposed to retinoids for 72 hr. The extent of granulocytic differentiation was assayed by measuring the oxidative burst potential via reduction of nitroblue tetrazolium (NBT)

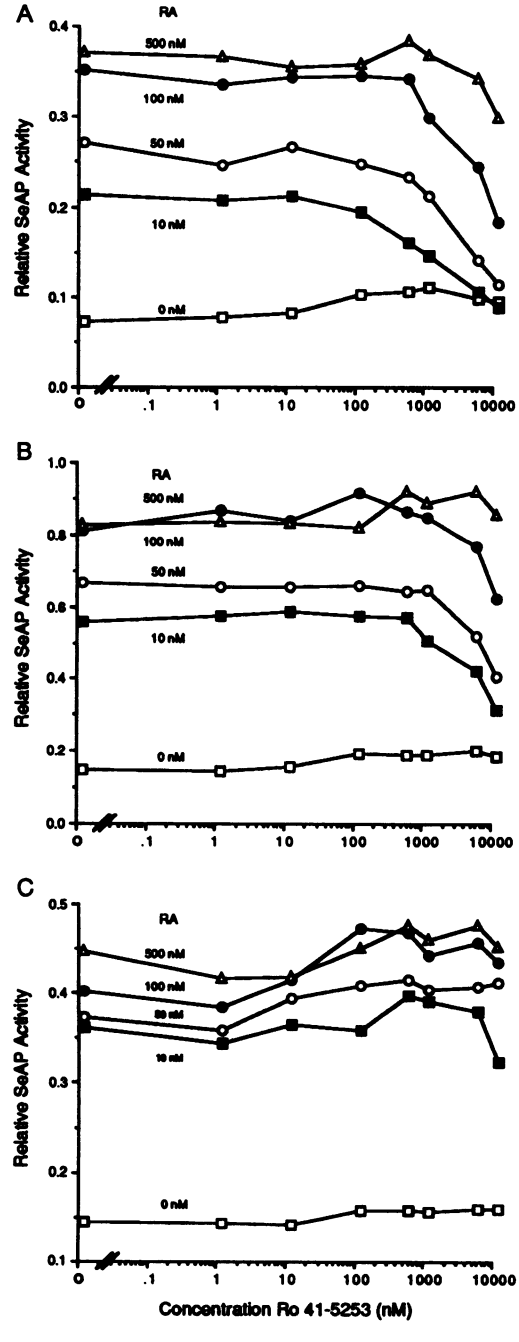


FIG. 1. Influence of Ro 41-5253 on transactivation induced by RA. (*A*) COS-1 cells were transfected with RARα-ER and the reporter plasmid vit-tk-SeAP. (*B*) Same as in *A* but with RARβ-ER. (*C*) Same as in *A* but with RARγ-ER. Twenty-two hours after transfection, RA at various concentrations was added to the cultures as follows: □, 0; ■, 10; ○, 50; ●, 100; △, 500 nM.

(Sigma) according to the method of Pick *et al.* (41). The amount of reduced NBT was measured photometrically in an automated plate reader.

Mouse Cell Cultures. C57BL/6J Ico spleen cell suspensions were cultured at 2×10^5 cells per ml, 0.2 ml per well in 96-well Costar trays, in Iscove's modified Dulbecco's medium (GIBCO) supplemented with antibiotics, 50 μM 2-mercaptoethanol, 10% FCS (Flow Laboratories), and 50 μg of *E. coli* lipopolysaccharide (LPS) per ml (Difco). At days 2 and 3 of culture, cells were pulsed with 1 μCi of [^{3}H]thymidine per well (Amersham) for 4 hr, harvested on glass fiber filters, and processed for β-scintillation counting. Retinoids were titrated into the cultures at time 0.

RESULTS

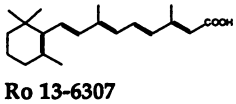
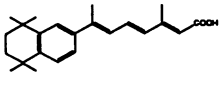
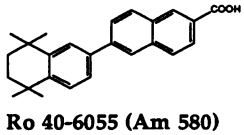
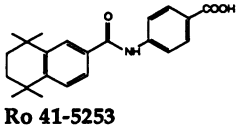
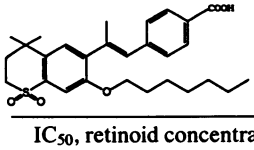
Ro 41-5253 As an Antagonist of Receptor Activation by Retinoids. Fig. 1 shows the dose-dependent antagonistic activity of Ro 41-5253 at different RA concentrations. This is reflected in the decreased induction of SeAP activity in COS-1 cells cotransfected with the different RAR-ER chimeric receptors plus reporter plasmid. The ED_{50} for RA alone is 10 nM for RARα-ER, 2 nM for RARβ-ER, and 1 nM for RARγ-ER (data not shown). The antagonist alone, in the absence of RA, did not induce appreciable activation of any of the receptors (maximum, <10% of the effect of RA at 500 nM).

The antagonist caused a dose-dependent reduction of the transactivation of RARα-ER (Fig. 1*A*). For a given RA concentration, 2- to 10-fold excess of Ro 41-5253 was needed to show an antagonistic effect. At the highest antagonist concentration (10 μM), the transactivation induced by RA (10 or 50 nM) could be completely suppressed. A 50- to 100-fold excess of antagonist was necessary for a reduction in activation of RARβ-ER and the suppression was never complete (Fig. 1*B*). In cells transfected with RARγ-ER (Fig. 1*C*), a modest effect was visible only at 10 nM RA and at 10 μM Ro 41-5253. The results concerning RARβ-ER and RARγ-ER reflect the low affinity of the retinoid for these receptors (40-fold and 55-fold lower than for RARα-DEF, respectively; see Table 1). These results show that Ro 41-5253 is a retinoid that antagonizes the transactivation of RARs by RA, having a high preference for RARα.

Three other retinoids were used as agonists instead of RA in the receptor transactivation assay. The binding characteristics of these compounds are shown in Table 1.

Fig. 2 shows the dose-dependent antagonism by Ro 41-5253 for different concentrations of these agonists (in COS-1 cells transfected with RARα). Ro 13-6307 activates the α receptor at a 20-fold lower concentration than RA (ED_{50}, 0.5 vs. 10 nM). Antagonizing the transactivation by Ro 13-6307 required a 20- to 100-fold excess of Ro 41-5253 (Fig. 2*A*). Ro

Table 1. Binding to RARα, RARβ, and RARγ (DEF domains) of RA and synthetic analogues

Retinoid	Binding (IC_{50}, nM) RARα	RARβ	RARγ
Retinoic acid 	14	14	14
Ro 13-6307 	8	6	7
Ro 19-0645 	460	26	190
Ro 40-6055 (Am 580) 	39	870	2700
Ro 41-5253 	60	2400	3300

IC_{50}, retinoid concentration required to inhibit 50% of specific RA binding.

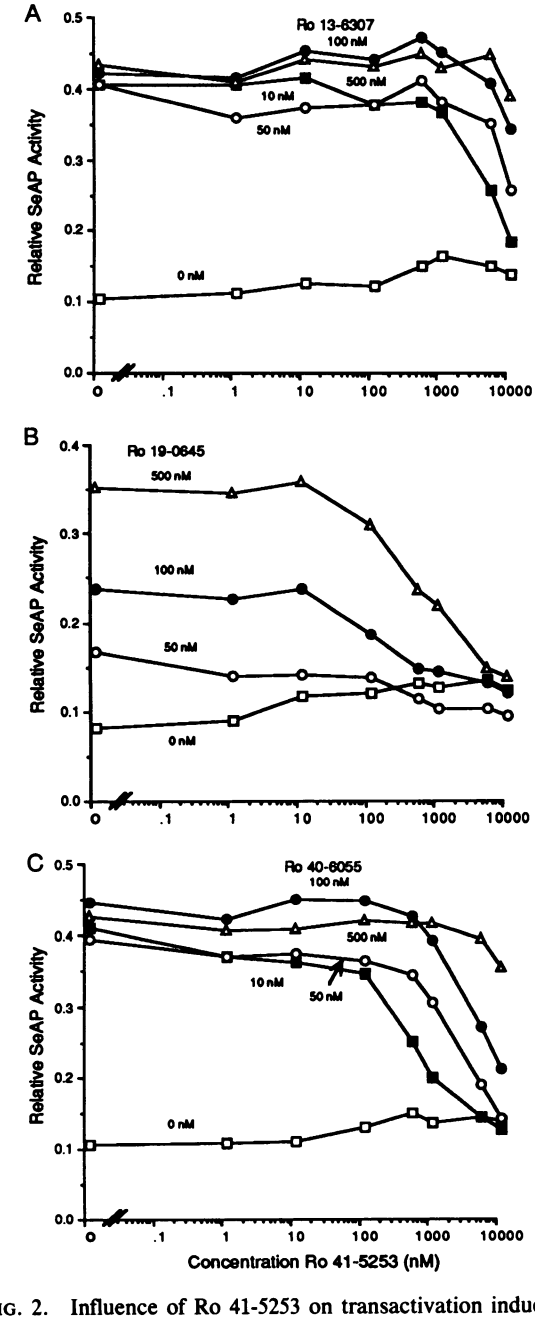


FIG. 2. Influence of Ro 41-5253 on transactivation induced by synthetic retinoids in COS-1 cells transfected with RARα and the reporter plasmid vit-tk-SeAP. Twenty-two hours after transfection, RA at various concentrations was added to the cultures as follows: □, 0; ■, 10; ○, 50; ●, 100; △, 500 nM.

19-0645 mediates a weaker activation of RARα-ER than RA (9-fold; ED_{50}, 90 nM). In this case, a lower concentration of antagonist was necessary for inhibition (equimolar at 100 nM agonist to 5-fold lower at 500 nM agonist) (Fig. 2*B*). Ro 40-6055 (also known as Am 580) (42) activates the α receptor with an ED_{50} of 2 nM. For this retinoid, a 10-fold excess of antagonist was required for a reduction in transactivation (Fig. 2*C*).

These results show that Ro 41-5253 is an antagonist not only for RA but also for other retinoids. The effective antagonist concentration is dependent on the potency of the agonist in RARα-ER transactivation. The lower the ED_{50} of the agonist (Ro 19-0645, 90 nM; RA, 10 nM; Ro 40-6055, 2 nM; Ro 13-6307, 0.5 nM), the higher the concentration of antagonist required for a reduction in transactivation (0.5- to 1-, 2- to 10-, 10-, 20- to 100-fold, respectively).

Ro 41-5253 Inhibits Retinoid-Induced Differentiation of the Promyelocytic Cell Line HL-60. RA induces differentiation of the promyelocytic cell line HL-60 along the granulocytic lineage (43). HL-60 cells express RARα but not RARβ (44) or RARγ (T. Uchida and P.L., unpublished data).

We have tested a large number of synthetic retinoids and found that the ability to induce differentiation is indeed a receptor-mediated event, since retinoids inactive in receptor transactivation were also inactive in this functional system. This applied also to Ro 41-5253. The selective RARα agonist Ro 40-6055 was inducing differentiation with a potency comparable to that of RA, although the maximal differentiation never exceeded 60% of that induced by RA; the potency of Ro 19-0645 was 100- to 500-fold lower. Ro 41-5253 prevented the retinoid-induced differentiation in all cases tested. The data relative to RA, Ro 40-6055, and Ro 19-0645 are shown in Fig. 3. For the strong inducers, a 100-fold excess of antagonist was required for inhibition, while equimolar concentrations were sufficient to counteract the effect of Ro 19-0645.

Ro 41-5253 Reverts Retinoid-Induced Inhibition of Mouse B-Cell Proliferation. RA and several synthetic retinoids inhibited, with different potency, the proliferation of mouse B cells induced by the polyclonal B-cell mitogen LPS. Inhibition never exceeded 80%. In fact, activated B cells were resistant to the inhibition (data not shown). Ro 41-5253 did not significantly affect B-cell proliferation (Fig. 4).

To assess the ability of Ro 41-5253 to counteract retinoid-induced inhibition, the antagonist was titrated into cultures exposed to different agonists at various concentrations. In all cases tested, the inhibition was reversed (Fig. 5). The concentration of antagonist yielding maximal reversion of the effect induced by an IC_{50} concentration of retinoids was in all cases 1 μM. Due to the different potency of the inhibitory retinoids, the ratios of agonist to antagonist ranged from 1:1000 to 1:10.

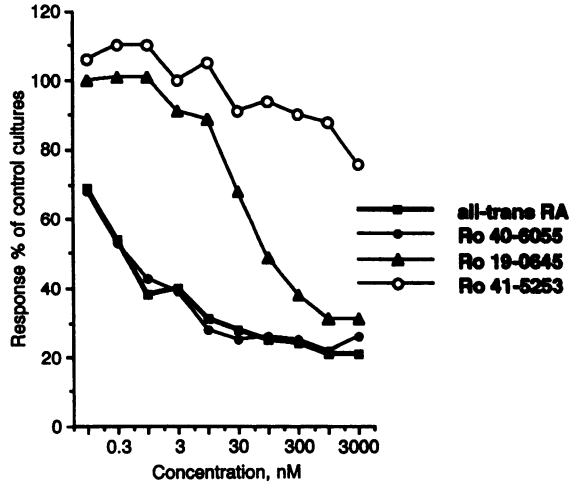


FIG. 4. Effect of RA and three synthetic retinoids, including Ro 41-5253, on LPS-induced mouse B-lymphocyte proliferation. Inhibition never exceeded 80% due to insensitivity to retinoids of preactivated cells, thereby making toxic effects of retinoids at high concentrations unlikely.

DISCUSSION

The complexity of the retinoid-dependent system of transcription factors can be dissected with the use of both selective retinoids activating exclusively or preferentially one given receptor, or by antagonists specifically interfering with one receptor. In the process of characterizing a large number of synthetic retinoids for their RAR-binding and transactivation properties, we found RARα-selective binders that were inactive in transactivation. The retinoid described here, Ro 41-5253, was inhibiting the transactivation of RARα by RA and other retinoids. An excess of antagonist over agonist was required for the inhibition, which was inversely related to the potency of the agonist in transactivation of RARα. Inhibition of transactivation of RARβ and RARγ was minimal. We may therefore consider Ro 41-5253 a selective RARα antagonist. We have no data about possible RARα isotype specificity.

Ro 41-5253 was able to counteract the RA-induced differentiation of the promyelocytic cell line HL-60. As for transactivation of RARα, the relative effective concentration of antagonist was depending on the potency of the agonist. The effectiveness of Ro 41-5253 in inhibiting the RA-induced differentiation indicates an involvement of RARα in the differentiation process. This could be expected as RARα is the only RAR expressed in this cell line (ref. 44; T. Uchida and P.L., unpublished data), and it was suggested by the possibility of restoring the RA sensitivity of a resistant HL-60 variant by transfection of RARα (45).

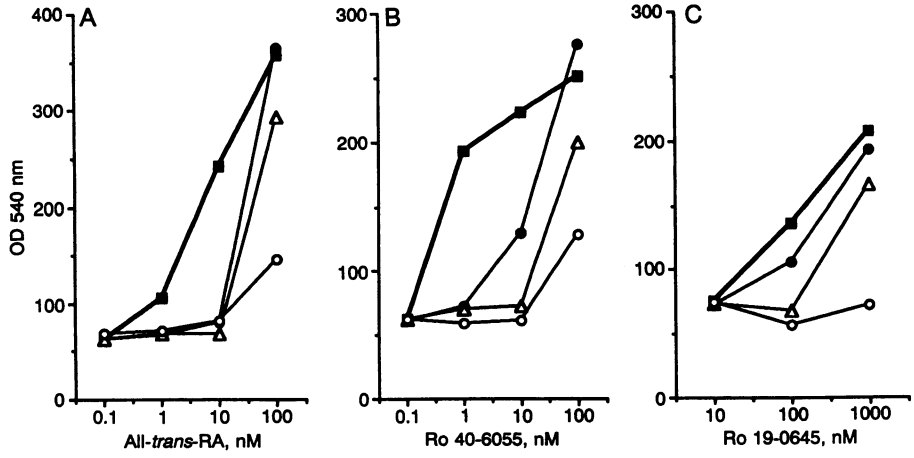


FIG. 3. Effect of Ro 41-5253 on retinoid-induced differentiation of the promyelocytic cell line HL-60. Viability of cultures was >95% by trypan blue exclusion. Ro 41-5253 concentrations: *A* and *B*, ■, 0; ●, 0.1; △, 1; ○, 10 μM; *C*, ■, 0; ●, 10; △, 100; ○, 1000 nM.

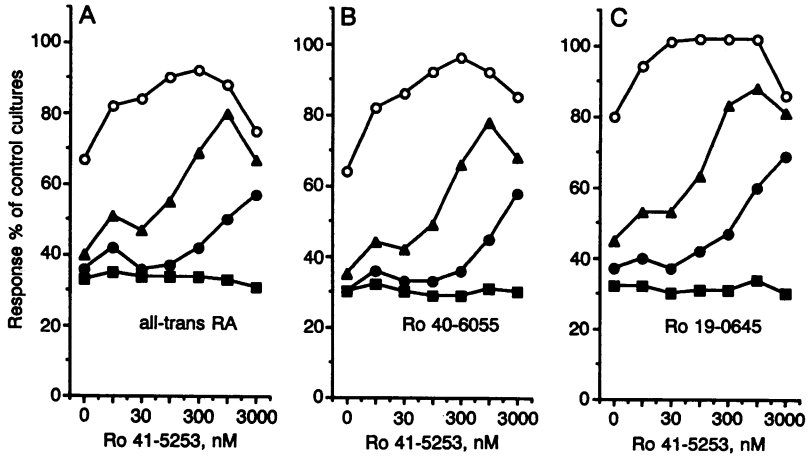


FIG. 5. Reversion by Ro 41-4253 of retinoid-induced inhibition of mouse B-cell proliferation. At an agonist concentration close to the IC_{50}, maximal reversion was always obtained with 1 μM antagonist. (*A* and *B*) ■, 100; ●, 10; ▲, 1; ○, 0.1 nM. (*C*) ■, 10; ●, 1; ▲, 0.1; ○, 0.01 μM.

Ro 41-5253 was also reversing the inhibitory effect of RA and other retinoids on mouse B-cell proliferation. Also in this system, an excess of antagonist was required depending on the potency of the agonist. B cells express RARα and RARγ but not RARβ (30). We had indications that RARα could be mediating this RA effect, since a selective RARα agonist, Ro 40-6055, was a potent inhibitor, and there was a correlation between the efficacy of a retinoid in RARα transactivation and its potency in inhibiting B-cell activation (L.F., P.L., and C.A., unpublished data). The reversion of the inhibition by a selective RARα antagonist supports RARα as a mediator of this RA effect.

We are well aware of the potential practical importance of selective receptor antagonists in diminishing or eliminating unwanted side effects of retinoids of therapeutic interest. In fact, the studies with Ro 41-5253 can be extended to crucial phenomena such as the hypervitaminosis A syndrome and RA-induced teratogenesis. On the other hand, we are also aware that targeting single retinoid receptors with specific ligands, with the aim of dissociating desired and undesired effects, will only be possible by improving our knowledge of receptor–function relationships, an achievement to which specific receptor antagonists will greatly contribute.

We thank Dr. P. Chambon (Faculté de Médecine, Strasbourg, France) for providing RARα-ER.CAS and RARβ-ER.CAS plasmids. The skilled technical assistance of K. Boscato, D. Ehrlich, A. Klem, C. Lacoste, B. Rutten, and M. Schweizer is gratefully acknowledged.

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