Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 339-345

# RETINOIC ACID AND SYNTHETIC ANALOGS DIFFERENTIALLY ACTIVATE RETINOIC ACID RECEPTOR DEPENDENT TRANSCRIPTION

Anders Åström[1], Ulrika Pettersson[1], Andree Krust[2], Pierre Chambon[2], and John J. Voorhees[1]

[1]Department of Dermatology, University of Michigan, Ann Arbor, MI 48109

[2]Laboratoire de Genetique Moleculaire des Eucaryotes du CNRS and U.184 INSERM, Faculte de Medecine, Strasbourg, France

Received September 20, 1990

**SUMMARY:** We have developed an assay where the potency of retinoids in retinoic acid receptor (RAR) mediated transcriptional activation can be rapidly evaluated. In this assay hRAR-$\alpha$, hRAR-$\beta$ and hRAR-$\gamma$ were expressed in CV-1 cells together with a reporter gene containing a retinoic acid responsive element (TRE$_3$-*tk*-CAT). Concentrations required to obtain half-maximum induction (ED$_{50}$) of CAT-activity were determined for several retinoids, e.g., all-*trans*-retinoic acid (RA), 13-*cis*-retinoic acid (13-*cis*-RA), arotinoid acid (TTNPB) and m-carboxy-arotinoid acid (m-carboxy-TTNPB, an inactive arotinoid analog). The ED$_{50}$ values for RA decreased in the order of RAR-$\alpha$ (24 nM) > RAR-$\beta$ (4.0 nM) > RAR-$\gamma$ (1.3 nM), while the ED$_{50}$ values for TTNPB and 13-*cis*-RA decreased in the order of RAR-$\alpha$ (6.5 nM, 190 nM) > RAR-$\gamma$ (2.3 nM, 140 nM) > RAR-$\beta$ (0.6 nM, 43 nM), respectively. No significant inductions were obtained when cells were treated with m-carboxy-TTNPB, even at 10 $\mu$M concentrations. The fold induction of CAT-activity for all compounds tested decreased in the order of RAR-$\alpha$ > RAR-$\beta$ > RAR-$\gamma$. © 1990 Academic Press, Inc.

Retinol (vitamin A) and retinoic acid (RA) are essential in the control of epithelial cell growth and cellular differentiation (1,2). It has been shown that retinoids prevent cancer in skin and have efficacy as agents in human malignant and premalignant cutaneous disorders (3). It has also been shown that retinoids cause growth inhibition in many hyperproliferating cell-lines, a feature that makes the compounds of fundamental interest as antitumor and antipsoriatic agents (1,3). Retinoids also play fundamental roles both in directing the spatial organization of cells during development and the generation of vertebrate limbs (4). The elucidation of the function of retinoids in these complex biological processes requires the identification of the specific components of the retinoid signal transduction system as well as the genes directly regulated by this system.

Several intracellular retinoic acid binding proteins have been identified, the cellular retinoic acid binding protein (CRABP) and the nuclear retinoic acid receptors (RAR) (1, 5-12). The role of the cytoplasmic retinoic acid binding proteins

0006-291X/90 $1.50
Copyright © 1990 by Academic Press, Inc.
All rights of reproduction in any form reserved.

in the transduction of retinoid signal remains undetermined. The direct effect of retinoids may be mediated by the RARs, which act as ligand-inducible transcriptional enhancer factors. These factors belong to the nuclear receptor superfamily of genes, which includes thyroid and steroid hormone receptors (13). Two different forms of RAR have been characterized in humans (RAR-$\alpha$ and RAR-$\beta$) (7-10) and a third receptor predominantly expressed in skin (RAR-$\gamma$) was recently identified (12). In a recent study (14), it was found that all analogs biologically active in F9 cells bound to RARs, while two of them did not bind to CRABP. These results suggest that retinoids must bind to RARs but not necessarily to CRABP in order to induce cell differentiation. On the other hand, little is known about the affinities of the individual RARs for different synthetic retinoic acid analogs. In the present study we have developed an assay where the potency of retinoids on retinoic acid receptor mediated transcriptional regulation can be evaluated.

## MATERIALS AND METHODS

### Materials

Retinoic acid and 13-*cis*-retinoic acid were purchased from Sigma Chemical Co. TTNPB (P-[(E)-2-(5,6,7,8,-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl] benzoic acid), m-carboxy-TTNPB,(m-[(E)-2-(5,6,7,8,-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl)-1-propenyl] benzoic acid), BASF-46928 (4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtalenyl)ethynyl]benzoic acid) and BASF-47011 ((E)-4-[2-(5,6,7,8-tetrahydro-7-hydroxy-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzyl alcohol) were gifts from BASF Aktiengesellschaft, while etretin (all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid and etretinate (all-*trans*)-9-(4-methoxy-2,3,6,-trimethylphenyl) -3,7-dimethyl-2,4,6,8-nonatetraenoic acid ethyl ester) were gifts from Hoffman LaRoche. Butyryl CoA and [$^{14}$C] Chloramphenicol were purchased from Sigma and Amersham, respectively.

### Cell transfections and CAT-assays

CV-1 cells were grown in Dulbecco's modified eagles medium (DMEM) containing 10% fetal calf serum. The day before transfection, cells were seeded on 35mm tissue culture dishes in DMEM containing 10% charcoal treated fetal calf serum (ChFCS) at a density of 1.5-2.0 x $10^5$ per dish. Cells were cotransfected using the calcium phosphate co-precipitation technique (15) with 0.6 $\mu$g of hRAR expression vectors (hRAR$\alpha$0, hRAR$\beta$0 or hRAR$\gamma$0), 1.2 $\mu$g reporter plasmid and 1.2 $\mu$g of a $\beta$-galactosidase expression vector (pCH110, Pharmacia) used as an internal control to normalize for variations in transfection efficiency. The reporter plasmid $(TRE)_3$-*tk*-CAT was constructed by ligating synthetic oligonucleotides encoding three palindromic thyroid hormone response elements (TRE; ref. 16) ($(TCAGGTCATGACCTGA)_3$) flanked by HindIII and BamHI sites on the 5'- and 3' ends respectively and cloned into the HindIII - BamHI cloning sites of pBLCAT2 (17). The construct was confirmed by sequencing. 24 h after transfection, cells were washed once with DMEM, and medium (DMEM, 10%ChFCS) containing different concentrations of retinoids dissolved in DMSO or just DMSO were added to the cells. 24 h later the cells were trypsinized and suspended in medium, pelleted and washed once with 40mM Tris-Cl, pH 7.6 containing 150mM NaCl and 1mM EDTA. Cell lysates were prepared by three consecutive freeze-thaw cycles (15) and CAT-activity was determined by the xylene extraction method essentially as described (18). $\beta$-Galactosidase activity was determined in cell-lysates as described (15).

## RESULTS AND DISCUSSION

**Retinoic acid-dependent transcriptional activation of TRE3-CAT by RAR-α, RAR-β and RAR-γ:**

CV-1 cells were transfected with RAR-α, RAR-β, and RAR-γ expression vectors, and the dose response effect of RA on the transcription of the reporter plasmid TRE3-*tk*-CAT was studied. As can be seen in Figure 1, there was a dose dependent increase in CAT activity for all three receptors. However, there were differences in the concentration required to give 50% of maximum induction among the three receptors (Table 1). RAR-α needed almost 20-fold higher concentrations of RA compared to RAR-γ , and 6-fold higher concentration compared to RAR-β to give half maximum induction of CAT activity. The $ED_{50}$ values for RA obtained in this study (Figure 1, table 1) are consistent with a recent report (19) were half maximum induction for the mouse RAR-β and RAR-γ were found to be 2 nM and 0.5 nM respectively. In another recent study (20) an $ED_{50}$ value of 50 nM was reported for the human RAR-α and in another report (9) a 10 fold higher dose was required to activate the reporter gene *vit-tk*-CAT by the chimeric receptor RAR-α-ER-CAS compared to RAR-β-ER-CAS, which also is consistent with what we find in this study. These differences in concentrations required to obtain half maximum induction of CAT activity by the receptors most probably reflect different affinities of each RAR for retinoic acid. This assumption is supported by two recent reports, one where RA was found to have a $K_d$ value of 10-15 nM for RAR-α (21) and another where a higher affinity of RA to RAR-β compared to RAR-α was observed (22).

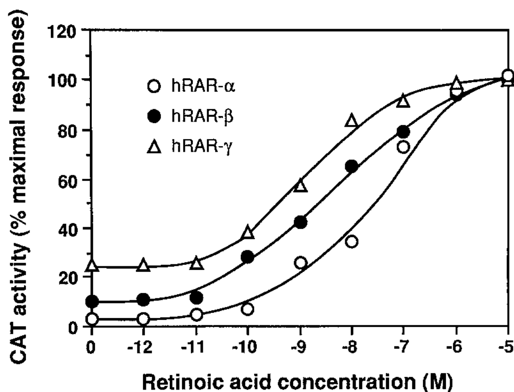


**Figure 1.** Retinoic acid-dependent transcriptional activation of TRE3-*tk*-CAT by RAR-α (o), RAR-β (●) and RAR-γ (Δ). CV-1 cells were cotransfected with expression vectors containing human RARs and pCH110 (a β-galactosidase expression vector) as an internal control. After transfection (24 h), cells were fed media containing increasing concentrations of RA as indicated. CAT activity was determined in cell lysates 48 h after transfection and normalized to β-galactosidase activity. CAT activity is expressed as per cent maximal induction.

**Table 1**
Concentrations of retinoids required to obtain half-maximal stimulation (ED50) of $TRE_3$-*tk*–CAT transcription by retinoic acid receptors (RARs) cotransfected into CV-1 cells

| COMPOUND | hRAR-α | hRAR-β | hRAR-γ |
|---|---|---|---|
| | | $ED_{50}$ (nM) | |
| Retinoic acid | 24.0 | 4.0 | 1.3 |
| 13-*cis* Retinoic acid | 190.0 | 43.0 | 140.0 |
| TTNPB | 6.5 | 0.6 | 2.3 |
| m-Carboxy TTNPB | n.i.[a] | n.i. | n.i. |
| BASF-46928 | 7.0 | 0.7 | 2.2 |
| BASF-47011 | >500 | 111.0 | >500 |
| Etretin | 69.0 | 480.0 | >500 |
| Etretinate | n.i. | n.i. | n.i. |

[a] n.i. = no induction. No significant induction was obtained at concentrations up to 10μM.

**Dose response of RAR-α, RAR-β and RAR-γ to different retinoids:**

CV-1 cells cotransfected with the RARs and the reporter gene were treated with increasing concentrations ($10^{-12}$ - $10^{-5}$ M) of different retinoids, and the $ED_{50}$ values were determined graphically. As can be seen in table 1, RA, TTNPB and BASF-46928 were the most effective compounds among those tested. However, $ED_{50}$ values for RAR-α and RAR-β were found to be lower when cells were treated with TTNPB and BASF-46928 compared to RA, while lower $ED_{50}$ values were obtained for RAR-γ when cells were treated with RA. RAR-β was found to be the most sensitive of the receptors in activation of CAT activity by TTNPB, BASF-46928, and BASF-47011, while RAR-γ and RAR-α were most sensitive in activation by RA and etretin, respectively. For two of the compounds tested, m-carboxy-TTNPB and etretinate, no significant induction was obtained even at 10 μM concentrations. These data clearly demonstrate differences in ligand specificity among the RARs. Differences in retinoid binding between RAR-α, RAR-β and RAR-γ was also suggested by the higher evolutionary conservation of the ligand binding domain (E region) for a given RAR subtype (RAR-α, RAR-β or RAR-γ ) when comparing human and mouse RARs than for all three RARs within a given species (12).

**Potency of RAR-α, RAR-β and RAR-γ in stimulating transcription by different retinoids:**

The $ED_{50}$ values obtained in the cotransfection assay (see above) most likely reflects the affinity of the receptors to different ligands, while the fold increase of CAT activity probably reflects the interaction of the ligand-receptor complex with the responsive element and its capability to induce transcription. The $ED_{50}$ values (Table 1) and the fold induction (Figure 2) for RA decreased in the order of RAR-α > RAR-β > RAR-γ . In other words RAR-γ seems to have the highest affinity to RA, but gives the lowest fold induction. Since it is important to optimize the amount of each RAR expression vector when discussing the relative efficiency by which RARs transactivate (23), we performed titrations using increasing amounts of RAR expression vectors (0.1 - 1200 ng). As can be sen in Figure 3, maximal induction was obtained for all three receptors when cells where transfected with 50 - 1200 ng of expression vector. Since optimal amounts of each RAR-expression vector (600 ng) was used in the transfection experiments (see material and methods), one can speculate that the activation function(s) of RAR-γ is intrinsically less efficient at stimulating transcription from $TRE_3$-*tk*-CAT and/or that other factors not present in CV-1 cells are required to yield maximum transcription. It has been reported that F9 cells and to a lesser extent CV-1 cells contains an activity that stimulates RAR-α binding to thyroid hormone responsive elements (24). For all compounds tested, the largest induction was obtained in cells transfected with RAR-α, while the lowest induction was obtained in cells transfected with RAR-γ (Figure 2). Transfection of the CV-1 cells with RAR-γ also consistently resulted in a higher background transcription in the absence of RA (about 2 fold, Figure 3.) compared to RAR-α and RAR-β. If this higher constitutive activity is an intrinsic property of the receptor or if remaining retinoids in the medium causes this effect remains to be determined. In agreement with our data (Figure 2), Giugere et. al. (19) recently observed a 4-fold lower induction of luciferase activity by mouse RAR-γ compared to human RAR-β in COS-1 cells. They also observed a higher constitutive activity when cells were transfected with mouse RAR-γ. When cells transfected with RAR-α were treated with TTNPB, BASF-46928 and etretin at saturating concentrations, we consistently obtained a significantly ($p<0.01$) lower (38-49%) induction of CAT-activity compared to the induction obtained by RA treatment (Figure 2), although TTNPB and BASF-46928 gave the lowest $ED_{50}$ values among the compounds tested. One possible explanation for this observation would be that if there is a transcriptional activation function (TAF) in the E/F region of the RARs, and if this function is dependent on RA, in the same manner as oestrogens are required for activation function TAF-2, present in the E/F region of the estradiol receptor (25, 26). Then, the lower fold induction obtained with TTNPB and BASF-46928 may reflect a lower efficiency of these compounds at inducing the TAF, even though their affinities for the ligand binding domain may be higher.

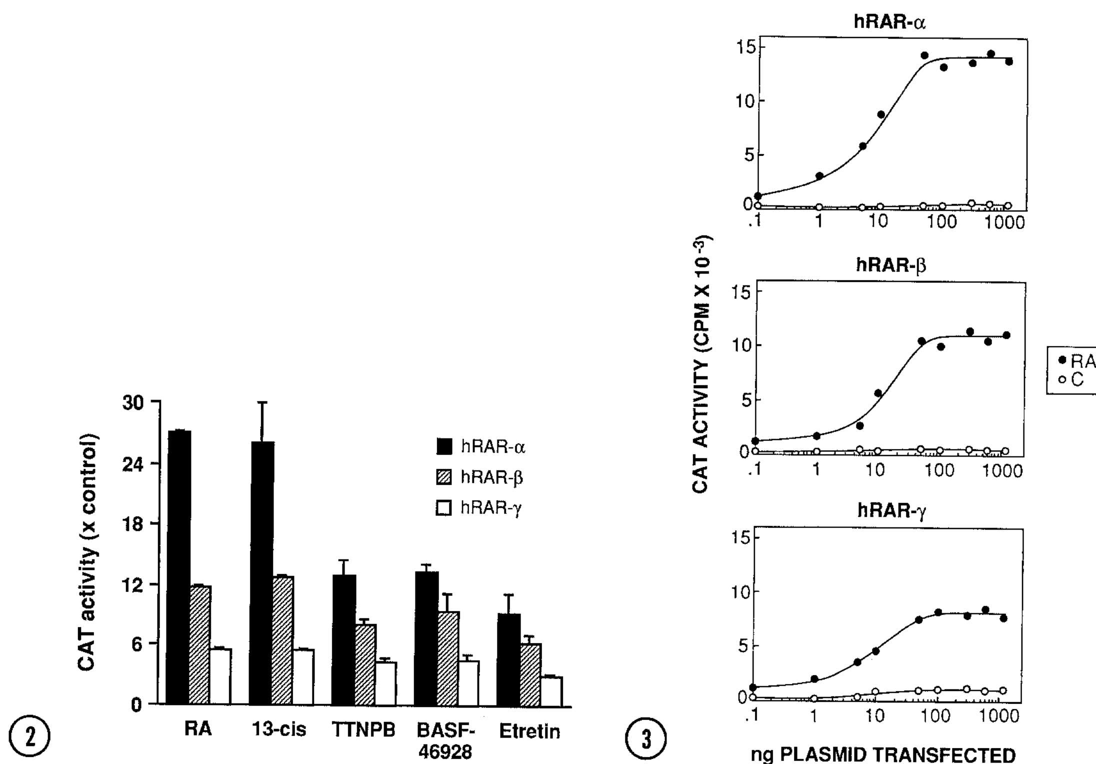


**Figure 2.** Induction of RAR transactivation by different retinoids. CV-1 cells were cotransfected with expression vectors containing human RARs and pCH110 (a $\beta$-galactosidase expression vector) as an internal control. After transfection (24 h), cells were fed media containing $10^{-6}$ M retinoid. CAT activity was determined in cell lysates 48 h after transfection and normalized to $\beta$-galactosidase activity. CAT activity is expressed as amount of induction in treated versus DMSO treated cells. The data represents mean ± SE of three experiments.

**Figure 3.** Transcriptional dose-response curves obtained by transfecting increasing amounts (0.1-1200ng) RAR expression vectors RAR-$\alpha$ (top) RAR-$\beta$ (middle) and RAR-$\gamma$ (bottom), together with 1.2 $\mu$g reporter plasmid (TRE3-*tk*-CAT) and 1.2 $\mu$g pCH110 (a $\beta$-galactosidase expression vector) into CV-1 cells. The plasmid ribobrobe pSP64 was used as a carrier to a total amount of 3.6 $\mu$g DNA in the transfection mixture. After transfection (24h), cells were fed media (C) or media containing $10^{-6}$ M RA (RA). The data represents the average of two experiments, and are expressed as cpm of acetylated chloramphenicol formed, normalized to $\beta$-galactosidase activity.

**Correlation with biological data:**

It has been demonstrated that there is a poor correlation between the affinities of some retinoids for RARs and observed biological activities (14). For example, TTNPB has been found to be 100 - 1000 fold more active *in vivo*, but still has affinities to F9 RARs (14) and HL60 RARs (27) similar to those of RA. Our data demonstrate that RAR-$\alpha$ and RAR-$\beta$ only have $ED_{50}$ values 3.7 and 6.7 times lower, respectively for TTNPB compared to RA, while the $ED_{50}$ value for RAR-$\gamma$ is even higher for TTNPB compared to RA. There are several possible explanations for this discrepancy: TTNPB may be more stable than RA in biological systems; TTNPB may induce other proteins involved in and/or down-regulate inhibitors of RAR mediated gene transcription; TTNPB may be more effective in inducing transcription mediated by responsive elements different from TRE.

In conclusion our data clearly demonstrate differences in ligand specificity among the RARs, suggesting the possibility of development of new retinoids that selectively activate transcription via one of the receptors. We also find significant differences among the receptors in their potential to activate transcription in this cotransfection system. Whether this reflects intrinsic differences between the three RARs activation functions in their ability to transactivate the TRE3-*tk*-CAT reporter gene or whether other factors are required to give maximal transcription are presently under investigation.

## ACKNOWLEDGMENTS

This work was supported by a Dermatology Foundation Research Fellowship (Anders Åström) and by a grant from the Lester I. Conrad Research Foundation.

## REFERENCES

1. Sporn, M.B., Roberts, A.B., and Goodman, D.S. (Eds.) (1984) *The Retinoids*, Vol. 1-2. Academic Press, New York.
2. Vahlquist, A., and Törmä, H. (1988) *Int. J. Dermatol.* **27**, 81-95.
3. Lippman, S.M., and Meyskens, F.L.Jr. (1989) *Pharmacol. Ther.* **40**, 107-122.
4. Maden, M. (1985) *Trends Genet.* **1**, 103-107.
5. Nilsson, M.H.L., Spurr, N.K., Saksena, P., Busch, C., Nordlinder, H., Peterson, P.A., Rask, L., and Sundelin, J. (1988) *Eur. J. Biochem.* **173**, 45-51.
6. Stoner, C.M., and Gudas, L.J. (1989) *Cancer Res.* **49**, 1497-1504.
7. Giguere, V., Ong, E.S., Segui, P., and Evans, R.M. (1987) *Nature,* **330**, 624-629.
8. Petkovich, M., Brand, N.J., Krust, A., and Chambon, P. (1987) *Nature,* **330**, 444-450.
9. Brand, N., Petkovich, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tillois, P., and Dejean, A. (1988) *Nature,* **332**, 850-853.
10. Benbrook, D., Lernhardt, E., and Pfahl, M. (1988) *Nature,* **333**, 669-672.
11. Zelent, A., Krust, A., Petkovich, M., Kastner, P., and Chambon, P. (1989) *Nature,* **339**, 714-717.
12. Krust, A., Kastner, P., Petkovich, M., Zelent, A., and Chambon, P. (1989) *Proc. Natl. Acad. Sci. USA.* **86**, 5310-5314.
13. Beato, M. (1989) *Cell,* **56**, 335-344.
14. Darmon, M., Rocher, M., Cavey, M-T., Martin, B., Rabilloud, T., Delescluse, C., and Shroot, B. (1988) *Skin Pharmacol.* **1**, 161-175.
15. Glass, C.K., Holloway, J.M., Devary, O.V., and Rosenfeld, M.G. (1988) *Cell,* **54**, 313-323
16. Luckow, B., and Schütz, G. (1987) *Nucleic Acids Res.* **15**, 5490.
17. Rosenthal, N. (1987) *Meth. Enzymol.* **152**, 704-720.
18. Seed, B., and Jeng-Yunn, S. (1988) *Gene,* **67**, 271-277.
19. Giugere, V., Shago, M., Zirngibl, R., Tate, P., Rossant, J., and Varmuza, S. (1990) *Mol. Cell. Biol.* **10**, 2335-2340.
20. Mangelsdorf, D.J., Ong, E.S., Dyck, J.A., and Evans, R.M. (1990) *Nature,* **345**, 224-229.
21. Cavey, M.T., Martin, B., Carlavan, I., and Shroot, B. (1990) *Anal. Biochem.* **186**, 19-23.
22. Hashimoto, Y., Kagechika, H., and Shudo, K. (1990) *Biochem. Biophys. Res. Commun.* **166**, 1300-1307.
23. Bocquel, M.T., Kumar, V., Stricker, C., Chambon, P., and Gronemeyer, H. (1989) *Nucleic Acids Res.* **17**, 2581-2595.
24. Glass, C.K., Lipkin, S.M., Devary, O.V., and Rosenfeld, M.G. (1989) *Cell,* **59**, 697-708.
25. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) *Cell,* **59**, 477-487.
26. Berry, M., Metzger, D., and Chambon, P. (1990) *Embo J.* **9**, 2811-2818.
27. Nervi, C., Grippo, J.F., Sherman, M.I., George, M.D., and Jetten, A.M. (1989) *Proc. Natl. Acad. Sci. USA.* **86**, 5854-5858.