Identification and Functional Separation of Retinoic Acid Receptor Neutral Antagonists and Inverse Agonists*

(Received for publication, June 10, 1996, and in revised form, July 3, 1996)

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Inverse agonists are ligands that are capable of repressing basal receptor activity in the absence of an agonist. We have designed a series of C-1-substituted acetylenic retinoids that exhibit potent antagonism of retinoic acid receptor (RAR)-mediated transactivation. Comparison of these related retinoid antagonists for their ability to repress basal RAR transcriptional activity demonstrates that the identity of the C-1 substituent differentiates these ligands into two groups: RAR inverse agonists and neutral antagonists. We show that treatment of cultured human keratinocytes with a RAR inverse agonist, but not a RAR neutral antagonist, leads to the repression of the serum-induced differentiation marker MRP-8. While RAR-selective agonists also repress expression of MRP-8, cotreatment with a RAR inverse agonist and a RAR agonist results in a mutual repression of their individual inhibitory activities, indicating the distinct modes of action of these two disparate retinoids in modulating MRP-8 expression. Our data indicate that RARs, like β2-adrenoceptors, are sensitive to inverse agonists and that this new class of retinoids will provide insight into the molecular mechanisms of RAR function in skin and other responsive tissues.

The concept of an inverse agonist, or negative antagonist, has grown out of studies of G-protein-coupled receptors where certain antagonists have been demonstrated to inhibit the activity of unliganded receptors (1–3). Recent evidence from transgenic mice with myocardial overexpression of the β2-adrenoceptor (4) has provided further support for a two-state model of ligand-regulated G-protein-coupled receptors. In this model, an equilibrium is postulated to exist between inactive receptors and spontaneously active receptors, which are capable of G-protein coupling in the absence of ligand. This model has provided the conceptual framework for the existence of agonists that shift the equilibrium toward active receptors, inverse agonists that shift the equilibrium toward inactive receptors, and neutral antagonists that themselves do not affect the receptor equilibrium but are capable of competitive antagonism of both agonists and inverse agonists.

In the nuclear hormone-steroid receptor superfamily, antagonists for the retinoic acid, progesterone/glucoorticoid, and estrogen receptor have been described (5–8). The anti-progesterone effects of RU-486 and the anti-estrogen effects of 4-hydroxytamoxifen have been demonstrated to have clinical utility in termination of pregnancy (9) and treatment of hormone-dependent breast cancer (10), respectively. Inverse agonists have not been previously reported for the nuclear receptor family. We have recently described a C-1-substituted acetylenic retinoic acid, AGN 193109, which exhibits potent antagonism of all-trans-retinoic acid (ATRA)1-mediated transactivation of RARα, RARβ, and RARγ (6) as well as blockade of retinoid-mediated topical irritation in animal models (11). Using a series of AGN 193109 analogs, which differ in their C-1 substituent, we demonstrate that these RAR antagonists can be differentiated on the basis of their ability to inhibit, or trans-repress, RAR basal transcriptional activity in the absence of exogenously added retinoid agonist. We show that a RAR inverse agonist, but not a neutral antagonist, is capable of regulating gene expression of a differentiation marker in cultured human keratinocytes in a manner that is distinct from that of a classical retinoid agonist. As such, RARs, like G-protein-coupled receptors, are sensitive to inverse agonists, and such activity may be amenable to the design of retinoids with unique therapeutic potential.

EXPERIMENTAL PROCEDURES

Transfections and DNA Constructs—For ATRA antagonism studies, 4 × 105 CV-1 cells (12-well Costar plate) were transiently transfected via calcium phosphate precipitation (12) with 0.7 μg of the reporter plasmid MTV-4-R5G-Luc containing four copies of the DR-5 RARE (5′-AGGGTTCCAGGAAAGACGT-3′) inserted into the HindIII site of the plasmid 3MTV-Luc (13), 0.1 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia Biotech Inc.), 0.01 μg of the plasmid pR5-hRXRα (14), and 0.05 μg of either pRS-RARα-P-GR (15), pcDNA3-RARβ-P-GR, or pcDNA3-RARγ-P-GR. RARβ-P-GR and RARγ-P-GR vectors were constructed via polymerase chain reaction-mediated mutagenesis of the P-box followed by insertion of the altered cDNAs into the mammalian expression vector pcDNA3 (Invitrogen). Receptors expressed from these plasmids contain DNA binding domains in which the P-box of the retinoid receptor (EGCRG) has been altered to that of the glucocorticoid receptor (GSKCV), allowing for RXR/RAR recognition of the R5G RARE. Eighteen hours after introduction of the DNA precursors, cells were rinsed with phosphate-buffered saline and fed with Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% activated charcoal-extracted fetal bovine serum (Gemini Bio-Products). Cells were treated for 18 h with 10 nM ATRA in conjunction with the compounds indicated in the figure. After rinsing with phosphate-buffered saline, cells were lysed and luciferase activity was measured as described previously (16). Luciferase values represent the mean ± S.E. of triplicate determinations normalized to β-galactosidase activity.

For analysis of ER-RAR chimeric receptor transactivation, 4 × 104 CV-1 cells (per well of a 12-well Costar plate) were transiently transfected via calcium phosphate precipitation with 0.5 μg of pERE-tk-Luc (containing the estrogen-regulated element of the Xenopus vitellogenin 1 The abbreviations used are: ATRA, all-trans-retinoic acid; RAR, retinoic acid receptor; RXX, retinoid X receptor; ER, estrogen receptor; tk, thymidine kinase; Luc, luciferase; HSV, herpes simplex virus; ERE, estrogen receptor response element; RARE, RAR response element.

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C-1-substituted Acetylenic Retinoids Are RAR Antagonists—

The structurally related acetylenic retinoids in Table I were tested for their ability to competitively bind the three RAR subtypes. All of the compounds exhibit high affinity binding to the RARs and no measurable binding to the RXRs. With the exception of AGN 192870 and 193840, these ligands do not transactivate the RARs as measured in CV-1 cells transiently transfected with either chimeric ER-RARs or full-length RARs using appropriate reporter constructs (data not shown). AGN 192870 and 193840 do exhibit partial agonist (approximately 30% compared with ATRA) activity at RARβ only (data not shown). As shown in Fig. 1, the ligands of Table I are effective RAR antagonists, inhibiting ATRA-mediated transactivation with the expected RARβ partial antagonist behavior of AGN 192870 and 193840. Determined IC50 values for these RAR antagonists (see Table I) are in agreement with their binding affinities (Kd) for the RARs.

Repression of Basal RAR Transcriptional Activity—By analogy with inverse agonists for the β2-adrenergic receptor, a RAR inverse agonist should inhibit RAR basal transactivation. We analyzed the ability of AGN 193109, the highest affinity RAR ligand of Table I, to repress the basal activity of chimeric ER-RAR receptors containing the DEF domain of RARα, -β, or -γ (30). Treatment of transfected cells and determination of luciferase activity were performed as described for ATRA antagonism studies (above).

For analysis of ligand regulation of RARγ–VP-16, CV-1 cells were transfected as outlined above with 0.5 μg of pERE-tk-Luc, 0.1 μg of pCH110, 0.1 μg of ER-RXRα expression vector, and 0.2 μg of the chimeric expression vector RARγ–VP-16. ER-RXRα contains the hormone binding domain (amino acids 181–458) of RXRα (14) fused downstream from the estrogen receptor A/B and DNA binding domains (20). RARγ–VP-16 has been previously described (21) and contains the activation domain of herpes simplex virus VP-16 fused to the N terminus of full-length RARγ. Transferring constitutive activity of RARγ–VP-16 is directed to pERE-tk-Luc by heterodimerization of the RARγ moiety to ER-RXRα bound to the ERE.

**RESULTS AND DISCUSSION**

A2 gene (17) inserted into the plasmid tk-luciferase (18), 0.1 μg of pCH110, and 0.05 μg of the SV40-based vector pECE (19) expressing chimeric ER-RAR receptors consisting of the estrogen receptor A/B and DNA binding domains fused to the DEF domain of RARα, -β, or -γ (20). Treatment of transfected cells and determination of luciferase activity were performed as described for ATRA antagonism studies (above).

For analysis of ligand regulation of RARγ–VP-16, CV-1 cells were transfected as outlined above with 0.5 μg of pERE-tk-Luc, 0.1 μg of pCH110, 0.1 μg of ER-RXRα expression vector, and 0.2 μg of the chimeric expression vector RARγ–VP-16. ER-RXRα contains the hormone binding domain (amino acids 181–458) of RXRα (14) fused downstream from the estrogen receptor A/B and DNA binding domains (20). RARγ–VP-16 has been previously described (21) and contains the activation domain of herpes simplex virus VP-16 fused to the N terminus of full-length RARγ. Transferring constitutive activity of RARγ–VP-16 is directed to pERE-tk-Luc by heterodimerization of the RARγ moiety to ER-RXRα bound to the ERE.

Ligand Binding Assays—Compounds were analyzed, as described previously (22), for their ability to competitively inhibit specific binding of [3H]ATRA or [3H]cis-retinoic acid to baculoviral expressed RARs and RXRs, respectively. Dissociation constants (Kd) were calculated via application of the Cheng-Prusoff algorithm (23). Values represent the average of three independent assays performed in duplicate ± the standard error of the mean.

Analysis of Cultured Human Keratinocytes—Human foreskin keratinocytes (passage 3), prepared as described previously (24), were maintained in keratinocyte growth medium (Clonetics) supplemented with 0.15 mM Ca2+ and hydrocortisone (1 μM). At 50–70% confluence, the medium was changed to keratinocyte growth medium without hydrocortisone. After 24 h, cells were treated with 10% serum (charcoal extracted) and retinoids every day for 5 days. Control cultures were treated with 10% serum and vehicle alone (0.02% dimethyl sulfoxide). Cell lysates were prepared in 300 μl of cell lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mg/ml phenylmethysulfonyl fluoride, 0.5 μg/ml leupeptin, and 2 μg/ml aprotinin) and centrifuged at 10,000 × g for 10 min. 30 μg of lysate was subjected to SDS-polyacrylamide gel electrophoresis (15%) and analyzed for MRP-8 protein by Western blot using a monoclonal anti-MRP-8 antibody, CF-145 (25).

**RESULTS AND DISCUSSION**

C-1-substituted Acetylenic Retinoids Are RAR Antagonists—

The structurally related acetylenic retinoids in Table I were tested for their ability to competitively bind the three RAR subtypes. All of the compounds exhibit high affinity binding to the RARs and no measurable binding to the RXRs. With the exception of AGN 192870 and 193840, these ligands do not transactivate the RARs as measured in CV-1 cells transiently transfected with either chimeric ER-RARs or full-length RARs using appropriate reporter constructs (data not shown). AGN 192870 and 193840 do exhibit partial agonist (approximately 30% compared with ATRA) activity at RARβ only (data not shown). As shown in Fig. 1, the ligands of Table I are effective RAR antagonists, inhibiting ATRA-mediated transactivation with the expected RARβ partial antagonist behavior of AGN 192870 and 193840. Determined IC50 values for these RAR antagonists (see Table I) are in agreement with their binding affinities (Kd) for the RARs.

Repression of Basal RAR Transcriptional Activity—By analogy with inverse agonists for the β2-adrenergic receptor, a RAR inverse agonist should inhibit RAR basal transactivation. We analyzed the ability of AGN 193109, the highest affinity RAR ligand of Table I, to repress the basal activity of chimeric ER-RAR receptors containing the DEF domain of RAR fused to the estrogen receptor A/B and DNA binding domains (see Fig. 2a). These chimeric receptors provide a higher unstimulated signal relative to full-length wild type RARs. Comparison of luciferase activity from cells transfected with the reporter plasmid ERE-tk-Luc alone with that of cells cotransfected with both reporter and receptor constructs indicated a stimulation of basal luciferase activity for ER-RARβ and ER-RARγ (Fig. 2b) but had a negligible effect upon the basal activity of ER-RARα cotransfectants. This refractoriness of ER-RARα to AGN 193109 treatment may be due to the relatively weak basal transactivation activity of this receptor in the absence of agonist.

We next tested the ability of AGN 193109 to repress the basal activity of a RARγ–VP-16 chimeric receptor where the constitutively active transactivation domain of herpes simplex virus (HSV) VP-16 is fused to the N terminus of RARγ (21). CV-1 cells were transfected either with ERE-tk-Luc and ER-RXRα or with ERE-tk-Luc, ER-RXRα, and RARγ–VP-16 (see Fig. 2a). As expected, luciferase activity in cells transfected with only ERE-tk-Luc and ER-RXRα is not regulated by AGN 193109 (Fig. 2c). Similarly, cells cotransfected with only ERE-tk-Luc and RARγ–VP-16 do not exhibit regulation of luciferase activity by AGN 193109 or RAR agonists (data not shown). As has been previously demonstrated (21), addition of RARγ–VP-16 to the transfection mixture results in a significant increase in the basal level of luciferase activity over that of ER-RXRα alone, consistent with heterodimerization of RARγ–VP-16 and ER-RXRα receptors at the ERE. While ATRA treatment resulted in a mild induction of activity, AGN 193109 treatment gave a strong, dose-dependent reduction of RARγ–VP-16 basal activity (Fig. 2c). Thus, AGN 193109 can potently repress the basal activity of RARs at different response elements, and this repressive activity can dominate the strong constitutive activity of a HSV VP-16 transactivation domain.

Functional Separation of RAR Inverse Agonists and Neutral Antagonists—We were interested to see if the trans-repression characteristics of AGN 193109 exhibited in the RARγ–VP-16

| R        | Kd (nM)  | IC50 (nM) |
|----------|----------|-----------|
| RARα     | 1.47 ± 0.07 | 87 ± 42   |
| RARβ     | 3.3 ± 0.6 | 32 ± 04   |
| RARγ     | 4.2 ± 0.9  | 52 ± 11   |
| AGN 192870 | 85 ± 4.5  | 42 ± 16   |
| AGN 193840 | 53 ± 5.7  | 52 ± 16   |
| AGN 193109 | 44 ± 7.5  | 29 ± 4.5  |
| AGN 193385 | 58 ± 4.9  | 18 ± 2    |
| AGN 193389 | 58 ± 4.9  | 27 ± 4    |
Identification of RAR Inverse Agonists

**Fig. 1. C-1 phenyl-substituted retinoids are RAR antagonists.** CV-1 cells were cotransfected with the luciferase reporter plasmid MTV-4(R5G)-Luc containing four copies of the DR-5 RARE R5G, the β-galactosidase expression plasmid pCH110 (Pharmacia), and RAR-P-GR expression plasmids for either RARα, β, or γ. These RAR-P-GR receptors contain glucocorticoid receptor P-boxes in their DNA binding domains and as such bind and transactivate as heterodimers with RXR at R-5-G DR-5 RAREs (15). Cells were treated for 18 h with 10 nM ATRA in conjunction with the compounds indicated in the figure. Luciferase values represent the mean ± S.E. of triplicate determinations normalized to β-galactosidase activity. Luciferase values for unstimulated transfected cells were 1441 ± 88, 1049 ± 113, and 822 ± 34 for RARα, β, and γ, respectively.

The assay would be shared by the four other RAR antagonists in Table I. Treatment of CV-1 cells transfected as described in Fig. 1c revealed two distinct categories of compounds (Fig. 2d). In contrast to that shown for AGN 193109, AGN 192870 and 193840 failed to repress RARγ-VP-16 activity even though they are potent and effective RARγ antagonists (Fig. 1). Similar to AGN 193109, 193385 and 193389 exhibit trans-repression of RARγ-VP-16, consistent with the concept that these RAR ligands are capable of active repression, or inverse agonism, in the absence of added RAR agonist. Thus the identity of the C-1 substitution is capable of differentiating neutral antagonists from inverse agonists. The two classes of compounds are characterized by distinct structural features in that the inverse agonists (AGN 193109, 193385, and 193389) have similarly bulky substituents (CH3, CF3, and Cl) at the 4-position of the phenyl ring while the neutral antagonists (AGN 192870 and 193840) have smaller substituents (H and F) at the same position.

Investigation of the effect of simultaneous treatment with both AGN 193109 and 193840 indicates the competitive nature of an inverse agonist and a neutral antagonist in trans-repression of RARγ-VP-16 transcriptional activity. As shown in Fig. 2c, the dose response of AGN 193109 is right shifted by cotreatment with two different concentrations of AGN 193840. Further, as shown in Fig. 2f, AGN 193840 antagonizes the AGN 193109-mediated trans-repression of RARγ-VP-16 in a dose-responsive manner. This transcriptional activation by the neutral antagonist AGN 193840 is right shifted using a higher constant dose of 193109, illustrating the competitive antagonism between the neutral antagonist and inverse agonist. Thus, RAR neutral antagonists and inverse agonists compete with one another for both binding as well as function through RARγ.

**RAR Inverse Agonist Function in Cultured Keratinocytes—** Retinoids inhibit the expression of differentiation markers in cultured normal human keratinocytes (27, 28) induced by confluence, elevated calcium, or serum treatment. We have recently shown that RAR-specific agonists inhibit the expression of the differentiation-specific gene MRP-8 (calgranulin A) in cultured keratinocytes. While expression of MRP-8 is not detectable in normal human skin, it is highly expressed in psoriatic epidermis and in cultured human keratinocytes differentiated with serum (25, 29, 30). Surprisingly, treatment of serum-differentiated human keratinocytes with the RAR inverse agonist AGN 193109 also mediates a dose-dependent repression of MRP-8 protein levels (Fig. 3a). In contrast to both RAR agonists and inverse agonists, the neutral antagonist AGN 193840 does not repress MRP-8 expression (Fig. 3a). Consistent with their competitive activities upon RARγ-VP-16 trans-repression (above), AGN 193840 cotreatment provides a dose-responsive antagonism of MRP-8 repression by both the RAR agonist TTNPB and the RAR inverse agonist AGN 193109 (data not shown). Interestingly, simultaneous administration of TTNPB together with AGN 193109, retinoids which as single agent treatments provide repression of MRP-8, results in a mutual antagonism of MRP-8 repression (Fig. 3b). Thus, a retinoid inverse agonist and a retinoid agonist are both capable of mediating repression of MRP-8 through apparently distinct mechanisms that are mutually exclusive in this system.

A growing body of evidence supports a general model of transcriptional activation of nuclear receptors involving ligand-mediated control of receptor interactions with both positive and negative associated factors (reviewed in Ref. 31). One possible mechanism underlying the inverse agonism of AGN 193109 may involve its ability to increase the interaction between the RAR and recently identified corepressor molecules (32, 33). Such modulation of corepressor-RAR interaction may provide a means to regulate gene expression directly at the RAR as well as through cross-talk with other nuclear receptors that share a common corepressor (34). Alternatively, a RAR inverse agonist may confer upon the RAR an inhibitory interaction with components of the core transcriptional machinery. AGN 193109 does not repress 12-O-tetradecanoylphorbol-13-acetate-stimulated AP-1 activity in HeLa cells. Further, this inverse agonist has no effect upon the ability of RAR/RXR heterodimers to bind DNA in vitro. Taken together, we propose that RAR inverse agonists function via shifting the equilibrium between RAR and RAR + corepressor toward the latter.

Given the level of relatedness among the nuclear hormone receptor family members and the apparent conservation of mechanistic paradigms associated with their regulation by

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2 S. Nagpal, manuscript in preparation.
3 D. Disepio, submitted for publication.
4 E. Klein, manuscript in preparation.
FIG. 2. **AGN 193109 is a RAR inverse agonist.**

*a*, schematic diagram of constructs used in transfection experiments. ER-RAR represents estrogen receptor A-B-C domain (*white boxes*) fused to the C-terminal D-E-F domain (*black boxes*) of either RARα, RARβ, or RARγ. ER-RXRα represents the C-terminal D-E domain of RXRα (*gray box*) fused to the A-B-C domain of the estrogen receptor. RARγ-VP-16 contains the transactivation domain of HSV VP-16 (VP16) and nuclear localization sequence (N) of SV40 T-antigen fused to full-length RARγ (*black boxes*). ERE-tk-Luc contains the estrogen-responsive regulatory element (*striped box*) of the vitellogenin A2 gene (17) inserted into the plasmid tk-luciferase (18). See “Experimental Procedures” for further details.

*b*, base-line activity of ER-RAR chimeric receptors is repressed by AGN193109. CV-1 cells were cotransfected with the luciferase reporter plasmid ERE-tk-Luc, the β-galactosidase expression plasmid pCH110 (Pharmacia), and either ER-RARα, ER-RARβ, or ER-RARγ. Cell were treated for 18 h with AGN193109 at the doses indicated in the figure. Luciferase values represent the mean ± S.E. of triplicate determinations normalized to β-galactosidase activity.

*c*, AGN 193109 repression of the constitutive transcriptional activity associated with the activation domain of herpes simplex virus VP-16 fused to RARγ. CV-1 cells were cotransfected with plasmids ERE-tk-Luc, pCH110, and ER-RXRα were treated with ATRA (○) or AGN 193109 (△) at the concentrations indicated in the figure. The weak activation by ATRA is consistent with conversion to 9-cis-retinoic acid and consequent activation of ER-RXRα homodimers (26). CV-1 cells cotransfected with ERE-tk-Luc, pCH110, and ER-RXRα and RARγ-VP-16 were treated with ATRA (○) or AGN 193109 (△). *d*, separation of RAR inverse agonists from neutral antagonists. CV-1 cells cotransfected with ERE-tk-Luc, pCH110, and ER-RXRα were treated with the compounds described in Table I.

*e* and *f*, competition of AGN 193109 inverse agonist activity by the neutral antagonist AGN 193840. CV-1 cells were transfected exactly as in panels *c* and *d*. Cells were simultaneously treated with both AGN 193109 and 193840 as described in the figure legends. Luciferase values in all panels of Fig. 2 represent the mean ± S.E. of triplicate determinations normalized to β-galactosidase activity.
FIG. 3. Mutual antagonism of the repression of MRP-8 in human keratinocytes by AGN 193109 and TTNPB. a, Western analysis of MRP-8 expression in keratinocytes cultured in 10% serum (charcoal extracted) and treated with either AGN 193109 or 193840 as indicated in the figure. b, Western analysis of MRP-8 expression in keratinocytes cultured in 10% serum (charcoal extracted) treated with vehicle alone (control, 0.02% dimethyl sulfoxide), 100 nM TTNPB, 1 μM AGN 193109, or both 100 nM TTNPB and 1 μM AGN 193109.

their respective ligands, it seems reasonable that inverse agonists for other family members may be identified. Our observations suggest that it is possible, with appropriate structural modifications, to design retinoid ligands with agonist, neutral antagonist, or inverse agonist properties, each with distinct biological properties.

Acknowledgments—We thank Dale Mais, Elain Berger, and Karen Flatten of Ligand Pharmaceuticals for performing the ligand binding assays. The CF-145 monoclonal anti-MRP-8 antibody was kindly provided by Veronica van Heyningen of MRC Human Genetics Unit, Edinburgh.

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J. Biol. Chem. 1996, 271:22692-22696.
doi: 10.1074/jbc.271.37.22692

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