A Novel Class of Retinoid Antagonists and Their Mechanism of Action*

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Retinoids regulate a broad range of biological processes through two subfamilies of nuclear retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Recently, we reported a novel type of retinoid acid antagonist (SR11335) and showed that this compound can inhibit retinoid acid (RA)-induced activation of a human immunodeficiency virus type 1 (HIV-1) promoter construct that contains a special RA response element (RARE). We have now further characterized the antagonism mediated by SR11335 and of newly synthesized structurally related compounds. Two compounds, SR11330 and SR11334, which are poor transactivators, also showed antagonist activities, inhibiting all-trans-RA (tRA) and 9-cis-RA. The retinoids inhibited transcriptional activation of RAR/RXR heterodimers effectively, while inhibition of RXR homodimers was less efficient. Inhibition was observed on several RAREs, including the TREpal, βRARE, apoAI-RARE, and CRBPI-RARE. In addition, the antagonists inhibited tRA-induced differentiation of HL-60 cells. The antagonist did not interfere with DNA binding of the receptors. In limited proteolytic digestion assays, SR11335 induced resistance of the receptors to proteolysis, but the pattern of the degradation was not altered from that induced by tRA, suggesting that these antagonists induce their biological effects by competing with agonists for binding to RARs, thereby preventing the induction of conformational changes of the receptors necessary for transcriptional activation.

Natural and synthetic vitamin A derivatives (retinoids) regulate a large variety of essential biological functions including cellular growth and differentiation (for reviews, see Refs. 1–3). The pleiotropic effects of retinoids are mediated by specific nuclear receptors that belong to the steroid/thyroid hormone receptor superfamily, a class of regulatory proteins that can function as ligand-responsive transcription factors (4). The retinoid receptors so far identified fall into two groups: the retinoic acid receptors (RARα, β, and γ) and the retinoid X receptors (RXRα, β, and γ). The receptors contain a number of distinct subdomains, a highly conserved cystine-rich domain necessary for sequence-specific DNA binding and a hydrophobic ligand-binding domain (reviewed in Ref. 4). Similar to steroid receptors, the retinoid receptors interact as dimers with specific DNA sequences, the retinoic acid response elements (RAREs), which are usually found in the promoter region of responsive genes. However, in contrast to steroid receptors that bind as homodimers to palindromic response elements, the RARs bind effectively as RAR/RXR heterodimers to a structurally diverse set of RAREs that contain a minimum of 2 hexanucleotide half-sites with the consensus sequence 5′-(A/G)GGTCA-3′ (5–8). The RXRs can also form heterodimers with a number of other hormone receptors such as thyroid hormone receptors, vitamin D3 receptors, and peroxisome proliferator-activated receptor (for reviews, see Refs. 4 and 9). In addition, RXRs can also act as homodimers in the presence of 9-cis-RA or RXR-selective synthetic retinoids (10, 11).

Recently, retinoids have been shown to enhance replication of several viruses such as the human immunodeficiency virus type 1 (HIV-1) and human cytomegalovirus (12, 16, 17). The retinoid response of the viruses appears to be controlled through elements in the long terminal repeat region of the viruses (12, 13). Indeed, RAREs have been identified in the long terminal repeat of HIV-1 (18, 19), cytomegalovirus (15), and human hepatitis B virus (14). In the case of HIV-1, a distinct RARE that contains two consensus half-sites arranged as a palindrome was located at nucleotides 348 to 328 of the long terminal repeat (18, 19). Both RXRα homodimer and RXRα/RXRα heterodimer are efficient activators of this RARE (18). Therefore, retinoid antagonists which can counteract retinoid activity, could provide a means of repressing viral replication by inhibiting the retinoid-dependent transcription and replication of the viruses in vivo. Besides providing important tools for deciphering mechanisms of retinoid action, the development of retinoid antagonists could therefore also be of importance because of their potential in the treatment of viral infections.

In general, the search for retinoid antagonists has met with limited success, and little is known about common structural features required for retinoid antagonists and their mechanisms of action. Recently, we reported that a novel synthetic retinoid, SR11335, repressed the retinoid-induced transcriptional activation of the HIV-1-RARE (18). These investigations were expanded to characterize further the retinoid antagonist activity of SR11335 and structurally related compounds as well as their mechanism of action. Here, we report that the retinoid antagonists, SR11335 and SR11330, can repress a broad spectrum of RAR/RXR heterodimer activities on a variety of RAREs and inhibit tRA-induced differentiation of HL-60 cells. Additionally, we studied the effects of the antagonists on the RAR/RXR heterodimer binding to RAREs and on ligand-induced
conformational changes of retinoid receptors by limited proteolytic digestion experiments.

MATERIALS AND METHODS

Retinoids—All-trans-RA was purchased from Sigma. 9-cis-RA was prepared by the method of Sakashita et al. (20). The intermediate ethyl 4-(4-formyl-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthryl)benzoate (FTTBA) was prepared by a Pd(0)-catalyzed Suzuki biaryl coupling (78%) of 2-bromo-5,6,7,8-tetrahydro-4-oxoanthrylm-5,5,8,8-tetramethylanthracene and 4-carboxyethoxybenzonic acid, followed by oximation of the anthridinium chromochrome, and then was used in synthesis to generate SR11330, SR11333, SR11334, SR11335, and SR11337. Treatment of FTTBA with CF3I and zinc in HCONMe2, followed by hydrolysis (KOH, aqueous ETOH; diluted HCl), afforded SR11330. Oxidation of SR11330 with CrO3 in aqueous H2SO4/acetone gave SR11334. Treatment of SR11334 with hydroxylamine hydrochloride and sodium acetate in ETOH, followed by diluted HCl, produced SR11333. Reaction of SR11330 with NaH and Me in HCONMe2, followed by hydrolysis (KOH, aqueous ETOH; diluted HCl), afforded SR11335. An alternate synthesis of SR11335 has also been reported (18). 4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-anthryl)benzoic acid (45) was acylated with treatment with (CF3CO)2O in pyridine, followed by hydrolysis (aqueous HCl), to give SR11336. FTTBA was oxidized under Baeyer-Villiger conditions (3-Cl-C6H4-CO3H in CH2Cl2), followed by base hydrolysis of the formate ester group (KOH, aqueous ETOH; diluted HCl) and acid hydrolysis of the ethyl ester with H2SO4 in aqueous acetic acid to give SR11337. All retinoids were fully characterized spectroscopically (IR, 1H NMR) and gave suitable elemental analyses. Retinoid stock solutions (10 mM) were made in a dimethyl sulfoxide:ethanol (1:1) mixture and maintained at –20°C.

Plasmids—The human and mouse retinoid receptor expression plasmids pCE- hRARα, -hRARγ, -hRXRα, -mRXRγ, and -mRXRγ and human estrogen receptor, pECE-ERγ, have been described previously (10, 22, 23, 24). The expression plasmids for chimeric receptors containing the DNA binding domain of estrogen receptor and the ligand binding domain of the retinoid receptor, i.e. pECE-ER-RARα, ER-RARγ, and ER-RXRα, have been described elsewhere (21). Construction of the reporter plasmids, HIV-1-RARE-tk-CAT, TRE-pal-tk-CAT, CRBP1-tk-CAT, pRARE-tk-CAT, apoAI-tk-CAT, and ERE-tk-CAT and have been described previously as well (18, 24–27).

Transient Transfection—CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were seeded at 5.0 × 10^4 cells per well in 24-well plates 16 to 24 h prior to transfection. A modified calcium phosphate precipitation procedure was used for transient transfection as described previously (10, 11, 29). Briefly, 100 ng of reporter plasmid, 200 ng of pECE-ER-RAR, apoAI-tk-CAT, and ERE-tk-CAT at 25°C for 20 min. The reaction mixtures were then loaded on a 5% agarose gel electrophoresis sample buffer, and then SDS-polyacrylamide gel electrophoresis was performed for min. The solution mixtures were then loaded on a 5% nondenaturing polyacrylamide gel containing 0.5× TBE (44.5 mM Tris borate, 44.5 mM boric acid, and 1 mM EDTA).

Limited Proteolytic Digestion Analysis—Protease digestion was carried out essentially as described previously (30–32). [35S]Methionine-labeled retinoid receptor was treated with trypsin for 30 min at room temperature. Retinoids were dissolved in a vehicle of dimethyl sulfoxide–ethanol 1:1 and final concentration of the vehicle was 1%. To μl of retinoid-treated receptor proteins, 2 μl of proteases in 10 μl Tis buffer (pH 7.6) was added and incubated for an additional 15 min at room temperature. The reaction was stopped by addition of 30 μl of SDS-polyacrylamide gel electrophoresis sample buffer and then SDS-polyacrylamide gel electrophoresis was performed to digest the retinoid antagonist for 12% (w/v) polyacrylamide gels. The gels were treated with a 25% isopropanol alcohol, 10% acetic acid aqueous solution for 1 h and followed by Amplify (Amersham) for 20 min. After drying with a vacuum drier for 50 min, the gels were autoradiographed.

HL-60 Cultures and Differentiation Measurements—The human promyelocytic leukemic cell line HL-60 cells were maintained in R1640 medium supplemented with 10% charcoal-treated fetal calf serum. 1 × 10^5 cells suspended in 100 μl of R1640 medium supplemented with 10% charcoal-treated fetal calf serum were plated in each well of 96-well plates and were grown in the presence or absence of variable concentrations of retinoids. After 3 days of incubation, 50 μl of fresh medium containing assigned amounts of retinoids were added, and incubation was continued for another 3 days. At the end of retinoid treatment, the cell proliferation was measured by the capacity of the cells to reduce nitro blue tetrazolium (Promega) according to the manufacturer’s instruction. The inhibition of cell proliferation by retinoids was used as a measurement of differentiation.

RESULTS

Retinoids with Low Transcriptional Activation Activities Show Antagonistic Effects—We conducted structure-activity studies on retinoids having low transcriptional activity, to identify the structural features required for retinoid antagonists. 4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-anthryl)benzoic acid (TTBA, SR3691), a retinoid having potent transcriptional activation activities for all three RARs (21) with EC50 values of 10 nM and comparable binding affinities (45) was selected as the starting structural template for these studies. The hydroxyl at the 4-position of the tetrahydroanthracene ring of TTBA was then replaced by a variety of groups containing different sizes, electronegativity, and polarity, including the following: 2,2,2-trifluoro-1-hydroxyethyl (SR11330), 2,2,2-trifluoro-1-(hydroxymino)ethyl (SR11331), 2,2,2-trifluorocarboxyl (SR11334), 2,2,2-trifluoro-1-methoxyethyl (SR11335), 2,2,2-trifluoroacetamido (SR11336), and hydroxy groups (SR11337) (Fig. 1). The transcriptional activities of these analogs were determined by using ER/RAR hybrid receptors with the reporter construct ERE-tk-CAT (21). In this series, SR11330 and SR11335 showed the lowest transcriptional activation activities, which were less than 10% of those of tRA at 10^{-8} M, whereas SR11334 showed moderate activity and the other analogs had significant activity, particularly with RARγ and RARγ (Fig. 2). The antagonist activities of these analogs on the RARα/RXRα heterodimer were tested first on the HIV-1-RARE in competition experiments with tRA. Of the substituents examined in this series, the 2,2,2-trifluoro-1-methoxyethyl group conferred the most potent antagonist activity, followed by analogs having the 2,2,2-trifluoro-1-hydroxyethyl (SR11330) and trifluorocarboxyl (SR11334) substituents (Fig. 3). Induction observed in the presence of 9-cis-RA was inhibited by the compounds to a similar extent (data not shown).

Antagonist Activity by RAR Inhibition—To characterize the mechanism of inhibition of the retinoid response by the antagonists, we studied whether the inhibition was restricted to certain combinations of retinoid receptors. SR11335 inhibited the RARα/RXRα heterodimer activity up to 80% (at 10^{-8} M). Inhibition was also obtained by SR11330 with less efficiency.
When only RAR\textsubscript{a} was cotransfected, the inhibition observed was close to that of the RAR\textsubscript{a}/RXR\textsubscript{a} heterodimer inhibition. In contrast, only about 30\% inhibition was observed for the RXR\textsubscript{a} homodimer activity (Fig. 4A). This suggested that the inhibition of the tRA-induced RAR\textsubscript{a}/RXR\textsubscript{a} heterodimer activity is mediated mainly through inhibition of the RAR\textsubscript{a}. Heterodimers with other RAR subtypes, i.e. RAR\textsubscript{b}/RXR\textsubscript{a} and RAR\textsubscript{g}/RXR\textsubscript{a}, were also repressed by the antagonists, but inhibition of the RAR\textsubscript{g}/RXR\textsubscript{a} heterodimer was less effective (Fig. 4B). To document that the inhibition observed with the antagonist was specific for the retinoid receptors, we also investigated the effect of the antagonists on estrogen receptor (ER). No significant effect on ER activation by estradiol was observed, whereas tamoxifen (10\(^{-6}\) M) had a profound effect (Fig. 4B). GR activity was not affected either (data not shown). RAR\textsubscript{s} also form heterodimers with the two other RXR subtypes, i.e. RXR\textsubscript{b} and RXR\textsubscript{g} (7, 8, 34). These heterodimers were similarly inhibited by the antagonists (Fig. 4C).

The currently identified RAREs show considerable structural differences (27, 28, 30, 41). To analyze whether SR11335 could differentially affect different RAREs, we compared its antagonistic effects on the CRBPI-RARE, the apoAI-RARE, both DR-2 type elements, and the \(b\)RARE, a DR-5. As shown in Fig. 5, the inhibition obtained was almost identical for those three RAREs and was also very similar to the inhibition seen with the HIV-1 RARE.

Antagonist Effects on DNA Binding—To obtain insight into the possible mechanisms by which these antagonists function, we investigated whether SR11335 and SR11330 could inhibit DNA binding of the retinoid receptors, possibly by inducing a receptor conformation that prevents DNA interaction. DNA binding was analyzed by gel retardation assay with \(\text{in vitro}\) translated receptors and radiolabeled response elements. SR11330 and SR11335 did not induce any noticeable changes in the binding of the receptors to the TREpal in the presence or absence (data not shown) of tRA. The same results were obtained with the \(b\)RARE and the HIV-1 RARE (data not shown).

Antagonist and Agonist Induce Overlapping Conformational Changes in the Receptors at Different Concentrations—Re-
**Fig. 4. Inhibition of retinoid receptor subtypes.** The TREpal-tk-CAT reporter was used to analyze receptor subtype selective inhibition. A, RARα and RXRα expression vectors were cotransfected with the reporter alone or in combination. B, RARβ or RARγ were cotransfected with RXRα. As a control, an ERE-tk-CAT vector was cotransfected with an ER expression vector. C, RARα and RARβ were also analyzed in the presence of RXRβ and RXRγ. Transfected cells were treated with 10⁻⁸ M tRA in the presence or absence of the indicated concentration of SR11330 (●), SR11335 (○), or Tamoxifen (▲). The activation obtained in the presence of 10⁻⁸ M tRA alone represents 100%. When only RXRα was transfected, 10⁻⁸ M 9-cis-RA was used, and 10⁻⁸ M estradiol was used when ER was transfected. Results of a representative experiment with duplicate measurements are shown.
We investigated whether the antagonists induced a distinct conformational change in the RARs. Limited proteolytic digestions with in vitro translated, [35S]methionine-labeled receptors were employed for those analyses. Labeled receptor was incubated alone or with tRA or SR11335, then digested for 15 min with a variety of proteases. Under these conditions, RARα alone was almost completely digested into fragments smaller than 14 kDa by trypsin. The receptor was only partially digested in the presence of tRA, resulting in the accumulation of a 30-kDa resistant fragment (Fig. 6A), as has been observed by others (31). The retinoid antagonist, SR11335, generated the same size of trypsin digestion-resistant fragment. However, the concentration of SR11335 required to induce the same degree of resistance was about 200-fold higher than that of tRA. Similar results were obtained with other proteases such as elastase and chymotrypsin. tRA also protected RARβ and RARγ from trypsin digestion and generated 33-kDa and 35-kDa fragments, respectively. In the presence of SR11335, a higher concentration (2 × 10^{-5} M) was required to obtain the same degree of resistance as seen with tRA (Fig. 6B). When only 10- or 100-fold higher concentrations of SR11335 were used (2 × 10^{-6} M, 2 × 10^{-7} M), little or no protection was seen (not shown). The observation that only relatively high concentrations of the antagonist were able to induce protected fragments is consistent with the apparently low affinity of these compounds for the RARs (the possible presence of retinoid agonist in the protein extracts could have only enhanced the activities of the antagonists in these assays). RXRα also generated a 19-kDa fragment that was resistant to chymotrypsin digestion in the presence of 9-cis-RA. However, SR11335 did not protect RXRα from the chymotrypsin digestion (Fig. 6C). These results are consistent with the weak effect of this retinoid in antagonizing activation by RXRα homodimers.

Antagonists Inhibit RA-induced Differentiation in HL-60 Cells—To evaluate the antagonistic effects of SR11335 in vivo, the human promyelocytic leukemia cell line, HL-60, was chosen since retinoid-induced differentiation has been well studied and characterized in these cells. HL-60 cells have been known to differentiate into granulocytes upon treatment with retinoids, and the retinoid effects are assumed to be mediated by RARα or together with RARβ since these receptors are expressed in this cell line (37, 38). HL-60 cells were incubated with the antagonists in the presence of tRA for 6 days, before measuring differentiation as a function of cell proliferation inhibition. As shown in Fig. 8, tRA induced differentiation in a dose-dependent manner. A concentration as low as 2.5 × 10^{-7} M SR11335 effectively reversed the growth inhibition (or differentiation) induced by 10^{-9} M and 10^{-8} M tRA. In the presence of 10^{-7} M tRA, the efficiency of repression of differentiation was less. The repression of differentiation was also obtained using two other retinoid antagonists, SR11330 and SR11334, although the effects were weaker (Fig. 7), demonstrating a good correlation between in vitro and in vivo results.

DISCUSSION

In the present study, we analyzed a related group of retinoids for their antagonistic effects on retinoid-induced transcriptional activation of nuclear retinoid receptors. Several of
these retinoids showed strong inhibition of RA-induced activation by various combinations of RAR/RXR heterodimers, but were less effective with RXR \( \alpha \) homodimers. It is likely that the inhibition observed on RXR \( \alpha \) homodimers represented inhibition of heterodimers formed between transfected RXR \( \alpha \) and endogenous RARs, since it has been shown that in the presence of both receptor species the formation of heterodimers is predominant in vivo. Inhibition was observed on all the retinoid response elements tested. Our transactivation studies, therefore, suggest that the antagonists function by competing with RAR agonists for RAR occupancy. This is consistent with the observation that antagonists induce a similar conformational change as the agonists determined by limited protease digestion, and, like the antagonist tRA, do not significantly affect DNA binding of the receptor heterodimers. However, the structural changes determined by the limited protease digestion pattern represent only a crude assessment of the ligand-induced changes in the receptor, since the conformation induced by agonists for receptor-mediated transcription activation must be distinct from the conformation induced by antagonists. The antagonist SR11335 did not induce a protease-resistant RXR fragment while 9-cis-RA did. This observation is also consistent with the transcriptional activation data, confirming that this antagonist functions through RARs.

It has recently been shown that RAR/RXR heterodimers interact with most RAREs with a defined polarity, such that RAR usually interacts with the 3' half-site of the response element (41). In this configuration, RAR can control the transcriptional activation of the heterodimer, preventing activation by RXR-selective ligands (43). By inhibiting the activation of RARs, retinoid antagonists may also be able to inhibit activation of the RXR component of the heterodimer. Such a mechanism could allow particularly efficient antagonistic effects by RAR-selective retinoid antagonists. The potential value for limiting responses to retinoids by RAR antagonists of this type is demonstrated by their ability to antagonize effectively tRA-induced growth inhibition/differentiation of HL-60 cells.

Kaneko et al. (39) reported two antagonists that inhibited tRA-induced differentiation of HL-60 cells. These compounds have the basic ring structure of the RAR-selective retinoidal benzamide 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamide/benzoic acid (Am580), which is a potent inducer of HL-60 cell differentiation, but have a 3-diamantyl-4-methoxyphenyl group in place of the tetrahydrotetramethylnapthalene ring of Am580. The bulky diamantyl group, which has a larger sterol volume than the corresponding 7-methylen- and 8,8-dimethylmethylene groups of Am580, permits binding to RAR\( \alpha \) but inhibits gene transcription. Apfel et al. (40) reported that (E)-6-[2-(4-carboxyphenyl)propenyl]-7-(1-heptylxy)-3,4-dihydro-4,4-dimethyl-2H-benzoazole3,1-dioxide (Ro41-5253) is a potent and selective antagonist of RAR\( \alpha \). This antagonist has the basic ring structure of Am580 and the potent RAR-selective retinoid (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl]benzoic acid (TTNPB, Ro13-7410), but has a sulfone group at the position corresponding to the 5-position of the tetrahydronapthalene ring and a heptyloxy group on this ring ortho to the bridge linking the aromatic rings. These studies indicate that steric bulk can be placed in regions corresponding to the hydrophobic head and tetraene side chain of the retinoid skeleton without loss of receptor binding activity. Our studies further define the constraints of binding and antagonism in the region of the tetraene chain using TTAB as the template for structural modification. The common structural feature of antagonists SR11335 and SR11330 are the 2,2,2-trifluoroethoxy group and a polar substituent at its 1-position. The 1-methoxy group of SR11335 produces more potent antagonism than the 1-hydroxyl group of SR11330, although neither compound binds as efficiently to RAR\( \alpha \) as Ro41-5253 does (data not shown). The trifluoro substituents appear to be essential for binding but not receptor activation because the 1-hydroxyethyl analog (SR11326) of SR11330 partially retains transcriptional activity. Replacement of the 1-methylene by a carbonyl group (SR11334) also confers partial agonist activity. Smaller hydrophobic groups at this ring position such as methyl do not decrease RAR\( \alpha \) transcriptional activity, whereas larger groups such as t-butyl are devoid of activity and poor binders to RAR\( \alpha \). Analogs having smaller polar groups such as hydroxyl (SR11337), amino, and hydroxymethyl are poor activators of RAR\( \alpha \), with activities comparable with that of the 1-hydroxyethyl analog. RAR\( \beta \) and RAR\( \gamma \) are more tolerant of substitutional modification at this position.

Our analyses indicate that the antagonists identified here interact with the same binding pocket as the agonists by blocking their access to the ligand binding domain. Interaction of the antagonist with the ligand binding domain leads to the same or a similar protease-resistant core as observed in the presence of agonist. However, it is clear that the agonist must induce an additional structural change allowing transcriptional activation not induced (or inhibited) by the antagonist. Further studies are needed to better understand the exact mechanism by which antagonists function at the receptor level.

The practical importance of retinoid antagonists has recently become apparent by the observation that several viruses are induced to replicate in the presence of retinoid agonists.
Whether antagonists can be designed that inhibit replication of viruses without causing a general block of the retinoid agonist activity remains to be explored.

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