AGN193109 Is a Highly Effective Antagonist of Retinoid Action in Human Ectocervical Epithelial Cells*

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Chapla Agarwalt, Roshantha A. S. Chandraratna§§, Alan T. Johnson§, Ellen A. Rorke***, and Richard L. Eckert†††

From the Departments of 1Physiology and Biophysics, 2Dermatology, 3Reproductive Biology, 4Biochemistry, and 5Environmental Health Sciences, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4970 and Retinoid Research, Departments of 1Chemistry and 1Biology, Allergan Pharmaceuticals, Irvine, California 92713

Retinoids are important physiological agents that regulate epithelial cell differentiation and proliferation. The importance of these agents in regulating growth, development, and differentiation has led to a search for new retinoid agonists and antagonists. In the present manuscript we show that AGN193109, a retinoid analog, is an efficient antagonist of retinoid action in human cervical epithelial cells. Treatment of ECE16-1 cells with natural or synthetic retinoids reduces cytokeratin K5, K6, K14, K16, and K17 levels, increases cytokeratin K7, K8, and K19 levels, increases retinoic acid receptor-β (RARβ) mRNA levels, suppresses proliferation, and alters cell morphology. Co-treatment with AGN193109 prevents these responses. Half-maximal and maximal antagonism is observed at a molar ratio of AGN193109:retinoid agonist of 1:1 and 10:1, respectively. When administered alone AGN193109 has no agonist activity. Thus, AGN193109, which binds to RARα, RARβ, and RARγ with Kd values = 2, 2, and 3 nM, respectively, but is unable to bind to the retinoid X receptors, is a highly active antagonist of retinoid action in ECE16-1 cells.

There is currently a great deal of interest in developing new retinoids for therapeutic application (1, 14, 36, 43). This interest has been fueled by the finding that retinoids are natural cancer preventive agents and that administration of retinoids for therapeutic application (1, 14, 36, 43). This interest has been fueled by the finding that retinoids are natural cancer preventive agents and that administration of retinoids can be used to treat existing cancer (2, 3, 15). Retinoids interact with the retinoid receptors that are ligand-activated, transacting transcription factors (4, 16, 17). The retinoid receptor family includes six major forms. Three of these, RARα, RARβ, and RARγ, display a preference for binding to all-trans-retinoic acid (TRA) (5-11, 17). The other three forms, RXRα, RXRβ, and RXRγ, bind to 9-cis-retinoic acid (9-cRA) (18, 19). Various retinoids have been described that interact selectively or specifically with the RAR or RXR receptors or with specific subtypes (α, β, or γ) within each class (1, 37, 38, 43). However, few antagonists of retinoid action have been described and they are mostly low affinity antagonists that have been poorly characterized at the receptor level (12, 20, 21, 39).

In the present study we describe the effects of a new retinoid, AGN193109, a specific and highly effective antagonist of RAR function (40), on the differentiation and proliferation of the ECE16-1 human cervical cancer cell line. ECE16-1 cells are an human papillomavirus type 16-immortalized cell line that was established by transfection of normal human cervical cells with the molecularly cloned human papillomavirus type 16 virus (22). They are a model for study of the early stages in cervical dysplasia and retinoid effects on cervical cell differentiation. These cells respond to retinoid stimulation with characteristic changes in proliferation rate, morphology, and marker gene expression (13, 22-25). The predominant receptor forms expressed in ECE16-1 cells are RARα, RXRα, and RARγ; however, RARβ is induced by retinoid treatment (37).

In the present study we demonstrate that AGN193109 is a highly active antagonist of retinoid action in ECE16-1 cells. We show that it is an efficient antagonist of retinoid-dependent changes in ECE16-1 cell morphology, gene expression, and proliferation, but has no intrinsic agonist activity.

MATERIALS AND METHODS

Cell Culture—ECE16-1 cells were routinely cultured in complete medium containing Dulbecco’s modified Eagle’s medium (D-MEM) (3:1), nonessential amino acids, 5% fetal calf serum, 2 mM triiodothyronine, 0.1 mM cholecalciferol, 2 μM l-glutamine, 1.8 × 10⁻⁴ M adenosine, and 10 nm epidermal growth factor (EGF). For proliferation experiments, the cells were shifted to defined medium (DM) containing Dulbecco’s modified Eagle’s medium (D-MEM) (3:1), 2 mM l-glutamine, nonessential amino acids, 0.1% bovine serum albumin, 1.8 × 10⁻⁴ M adenosine, 5 μg/ml transferrin, 2 mM l-glutamine, 50 μg/ml ascorbic acid, 100 μg/ml streptomycin, 100 units/ml penicillin, and 50 μg/ml gentamycin (26). DM was supplemented with EGF and/or retinoids as indicated.

Retinoids—TRA and 13-cis-retinoic acid (13-cRA) were obtained from Sigma, and 9-cRA, AGN193109, and TTNPB (27, 41) were synthesized in the Department of Chemistry, Allergan, Inc. Retinoid stocks were dissolved in dimethyl sulfoxide (Me₂SO) at 1000 μM concentrations and the DMSO concentration in the medium did not exceed 0.1% (28, 29).

Cell Proliferation Studies—ECE16-1 cell proliferation studies were performed exactly as described previously (23, 25, 26). Cells (10,000/cm²) were seeded in complete medium and allowed to attach overnight. The cells were then shifted to DM, allowed to equilibrate for 24 h, and treatment was initiated by addition of fresh DM or DM containing EGF or retinoid as indicated. After 3 days of daily treatment with retinoid, the cells were harvested with 0.025% trypsin, 1 mM EDTA, fixed in isotonic buffer containing 4% formaldehyde, and counted using a Coulter counter (26).

Protein and Nucleic Acid Methods—Pd[y(A)] RNA was prepared, electrophoresed, transferred to nylon membrane, and hybridized with 3²P-labeled cDNAs encoding gyceraldehyde-3-phosphate dehydrogenase.
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RESULTS

Retinoid Effects on Cell Morphology—Fig. 1 shows the morphology of untreated ECE16-1 cells (A) or cells treated with 10 nM TTNPB (B), 10 nM TTNPB + 100 nM AGN193109 (C), or 100 nM AGN193109 (D). TTNPB, a potent RAR-specific agonist, produces significant changes in cell morphology which are completely reversed by addition of a 10-fold molar excess of AGN193109. Although it efficiently antagonizes TTNPB effects, AGN193109 alone produces no change in ECE16-1 cell morphology. Dose-response curves indicate that the AGN193109 inhibits the TTNPB-dependent morphological change in a concentration-dependent manner.

AGN193109 Antagonizes Retinoid-dependent Growth Suppression—ECE16-1 cells remain quiescent in defined medium (Fig. 2A, open circle) and addition of 10 nM TTNPB prevents this proliferation. Addition of increasing concentrations of AGN193109 in the presence of 10 nM TTNPB suppresses the TTNPB-dependent suppression of proliferation (filled circles). The growth suppression is half-reversed by 10 nM and completely reversed by 100 nM AGN193109. Although it can antagonize the effects of TTNPB, 1 μM AGN193109 given alone has no effect on cell proliferation (filled triangle).

The ability of AGN193109 to antagonize the effects of TTNPB could be related to the fact that TTNPB is an RAR-specific ligand that does not interact with RXR receptors (Table I). We therefore determined whether AGN193109 could reverse the effects of tRA, 13-cRA, and 9-cRA. These retinoids interact with RAR or both RAR and RXR receptors (Table I). As with TTNPB, AGN193109, when present at a 10-fold molar excess, completely reverses the growth suppressive effects of tRA, 13-cRA, and 9-cRA (Fig. 2B).

AGN193109 Effects on Biochemical Markers of Retinoid Action—We next determined whether AGN193109 could antagonize retinoid effects on expression of retinoid-responsive marker genes. Cytokeratin proteins form the intermediate filaments in epithelial cells and are important markers of retinoid action in cervical epithelial cells (22, 33–35). As shown in Fig. 3A, ECE16-1 cells grown in retinoid-free medium express cytokeratins K5, K6, K7, K8, K13, K14, K16, K17, and K19 (A). Treatment with 10 nM TTNPB suppresses K5, K6, K14, K16, and K17 levels and increases K7, K8, and K19 levels (Fig. 3B). Simultaneous treatment with 100 nM AGN193109 eliminates the effects of 10 nM TTNPB (Fig. 3C). No changes in keratin expression are observed when cells were incubated with 0.01–1000 nM AGN193109 alone (not shown). We have shown previously that changes in keratin mRNA level mediate changes in keratin protein level (13, 22, 35). As a representative keratin, we show the effects of each treatment with the level of mRNA encoding K6 (Fig. 3B). AGN193109 (100 nM) has no effect and 10 nM TTNPB suppresses K6 mRNA levels. The TTNPB-dependent suppression is completely inhibited when cells are incubated with 10 nM TTNPB plus a 10-fold molar excess of AGN193109. AGN193109 also inhibits the retinoid-dependent change in the level of mRNA encoding the other keratins (not shown).

DISCUSSION

AGN193109 Antagonizes a Range of Responses in ECE16-1 Cells—Retinoids produce multiple effects on tissues and cells and different classes of retinoids can produce differing effects.
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#### TABLE I

| Compound   | Structure | RAR (Kd) | RXR (Kd) |
|------------|-----------|----------|----------|
| all-trans-RA | ![Structure](image) | 15 | 13 | 18 |
| 13-cis-RA | ![Structure](image) | 90 | 112 | 141 |
| 9-cis-RA | ![Structure](image) | 7 | 7 | 17 |
| TTNPB | ![Structure](image) | 30 | 3 | 2 |
| AGN193109 | ![Structure](image) | 2 | 2 | 3 |

AGN193109 Is an Efficient Antagonist with No Agonist Activity—Several problems can be encountered with receptor antagonists. First, they may have partial agonist activity. This can complicate interpretation of their effects in in vivo systems. To determine whether AGN193109 has intrinsic agonist or antagonist activity, we evaluated its effects on ECE16-1 cell function in the absence of added retinoid agonist. Concentrations of AGN193109 ranging from 0.01 to 1000 nM produced no detectable changes in cell morphology, cell proliferation, or retinoid-responsive marker gene expression. Based on these results we conclude (i) that AGN193109 is a highly effective antagonist of retinoid action in these cells and (ii) that it has not intrinsic agonist activity.

Second, antagonists may have a low affinity for the target receptor. Such ligands must be used at extremely high levels to accomplish the antagonism. AGN193109, however, has an affinity for the RARs of 2-3 nM. Considering that the receptor affinities of tRA, 13-cRA, and 9-cRA range from 7 to 141 nM and TTNPB has affinities ranging from 20 to 51 nM, AGN193109 is a high affinity ligand. This is reflected in its potency at preventing biological end responses. The compound is maximally effective at inhibiting retinoid agonist activity when present at a 10-fold molar excess and is half-maximally effective when present in equal molar amounts. Thus, AGN193109 is a high affinity antagonist.

Natural Ligands That Interact with RAR and RXR Receptors—Our initial studies showed that the synthetic retinoid, AGN193109, which interacts solely with RAR receptors, is effective at antagonizing the effects of the RAR-specific retinoid agonist, TTNPB. However, it was unclear whether the activity of natural ligands would be inhibited by AGN193109. We therefore tested the ability of AGN193109 to antagonize the effects of tRA, 13-cRA, and 9-cRA. 13-cRA, 9-cRA, and tRA interact with RARs, while 9-cRA also has a high affinity for RXRs. The results show that AGN193109 is an effective antagonist of the activity of each of these ligands. Thus, although AGN193109 only interacts with the RAR forms, it can also antagonize the effects of an agent (9-cRA) that interacts with relatively high affinity with both RARs and RXRs. Since AGN193109 does not interact with the RXRs, it is not likely that it directly prevents 9-cRA from interacting with RXR receptor. Moreover, RXR-specific ligands are relatively inactive in ECE16-1 cells (37). Since 9-cRA can efficiently bind to RARs, it is likely that AGN193109 is inhibiting 9-cRA effects mediated via RARs.

Receptor antagonists can be extremely useful ligands, both for understanding mechanism of action and for designing new therapies. In the present manuscript, we show in ECE16-1 cells, a human ectocervical epithelial cell line, that AGN193109...
is an extremely active high affinity antagonist of retinoid action that appears to have no intrinsic retinoid agonist activity.

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