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trans-Activation and Repression Properties of the Novel Nonsteroid Glucocorticoid Receptor Ligand 2,5-Dihydro-9-hydroxy-10-methoxy-2,2,4-trimethyl-5-(1-methylcyclohexen-3-y1)-1*H*-[1]benzopyrano[3,4-*f*]quinoline (A276575) and Its Four Stereoisomers

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ABSTRACT

Glucocorticoids are potent anti-inflammatory and immunosuppressant agents. However, they also produce serious side effects that limit their usage. It has been proposed that anti-inflammatory properties of glucocorticoids are caused mostly by repression of activator protein 1- and nuclear factor κβ-stimulated synthesis of inflammatory mediators, whereas most of their adverse effects are associated with *trans*-activation of genes involved with metabolic processes. Our laboratories have sought to discover novel glucocorticoid receptor (GR) ligands that have high repression but low *trans*-activation activities. We describe here cellular properties of 2,5-dihydro-9-hydroxy-10-methoxy-2,2,4-trimethyl-5-(1-methylcyclohexen-3-y1)-1*H*-[1]benzopyrano[3,4-*f*]quinoline (A276575) and its four enantiomers. Similar to dexamethasone, A276575 exhibited high affinity for GR and potently repressed interleukin (IL) 1β-stimulated IL-6 production in human skin fibroblasts, prostaglandin (PG) E_2 production in A549 human lung epithelial cells, and concanavalin A-induced monocyte proliferation. In contrast to dexamethasone, A276575 caused smaller induction of aromatase activity in human skin fibroblasts and antagonized dexamethasone-induced activation of an mouse mammary tumor virus–glucocorticoid-response element (GRE) reporter gene construct. Among the four enantiomers of A276575, the two (−)-enantiomers showed 10- to 30-fold higher affinities for GR than their respective (+)-enantiomers. Both (−)-Syn and (−)-Anti enantiomers of A276575 were potent inhibitors of IL-1β–stimulated PGE2 production in A549 lung epithelial cells; unexpectedly, however, only the (−)-Anti enantiomer inhibited regulated on T-cell activation, normal T-cell expressed and secreted (RANTES) production in A549 cells. In summary, A276575 is a novel, nonsteroidal GR ligand that possesses high repression activities against inflammatory mediator production but has lower GRE *trans*-activation activities than traditional steroids. Differential repression of RANTES and PGE2 production in a cell by the two (−)-enantiomers of A276575 illustrates the complexity of repression by GR.

Glucocorticoids (GCs) are highly effective and frequently prescribed anti-inflammatory and immunosuppressive agents for the treatments of rheumatoid arthritis, asthma, and transplantation (Schimmer and Parker, 1996). However, chronic GC usage is known to cause serious side effects such as osteoporosis, hyperglycemia, hypertension, hypothalamic-pituitary axis suppression, growth retardation, behavioral changes, and fat redistribution. These serious side effects have hampered the use of GCs for the treatment of inflammatory diseases. Therefore, it is highly desirable to identify novel GC ligands that retain anti-inflammatory activities of the steroids but have less undesirable side effects.

Glucocorticoid receptor (GR) is a member of the intracellular receptor superfamily of ligand-activated transcription

ABBREVIATIONS: GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; AP-1, activator protein 1; NF-κB, nuclear factor κB; A276575, 2,5-dihydro-9-hydroxy-10-methoxy-2,2,4-trimethyl-5-(1-methylcyclohexen-3-y1)-1*H*-[1]benzopyrano[3,4-*f*]quinoline; Dex, dexamethasone; MMTV, mouse mammary tumor virus; EIA, enzyme immunoassay; PGE2, prostaglandin E2; HSF, human skin fibroblast; IL, interleukin; PR, progesterone receptor; Prog, progesterone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; ConA, concanavalin A; RANTES, regulated on T-cell activation, normal T-cell expressed and secreted; RT-PCR, reverse transcriptase-polymerase chain reaction; COX-2, inducible cyclooxygenase.

factors (Karin, 1998). GCs binds GR in the cytosol and the GR-ligand complex translocates into the nucleus, where receptor-ligand complex dimerizes, binds to specific DNA sequences termed glucocorticoid-response elements (GRE), and increases the transcription of a number of genes involved with gluconeogenesis, fatty acid metabolism and metabolic processes. This GRE-activation activity has been implicated in many of the adverse events associated with GC therapy. In addition to *trans*-activation, GCs also inhibits the synthesis of a number of pro-inflammatory mediators by interfering with AP-1- or NF-κB–directed proinflammatory gene transcription (Cato and Wade, 1996). The mechanism of repression is probably via direct protein-protein interaction and does not involve *trans*-activation (Yang-Yen et al., 1990; Ray and Prefontaine, 1994). In some cells, GCs also induces IκB synthesis (Auphan et al., 1995; Scheinman et al., 1995). Evidence for a genetic dissociation between *trans*-activation and repression activities includes mutational studies of GR in vitro and transgenic mice expressing dimerization mutants in vivo (Caldenhoven et al., 1995; Reichardt et al., 1998). In addition, synthetic glucocorticoids that dissociate *trans*-activation and AP-1 *trans*-repression have also been described previously (Vayssiere et al., 1997).

Our laboratories have developed a new series of high affinity GR ligands, such as A276575 (Fig. 1). Unlike traditional GCs, such as dexamethasone (Dex) and prednisolone, these compounds do not contain the terpenoid structure. Although they repress proinflammatory mediator synthesis in cells, they show antagonistic activity at the MMTV-GRE reporter gene assays and lower activation of GRE-regulated aromatase enzyme induction in nontransfected human skin fibroblast cells. We describe here in vitro properties of A276575. A276575 contains two chiral centers and is a mixture of four enantiomers. To better understand the stereoselectivity of their interaction with GR, we synthesized all four of the enantiomers and characterized their GR activities as well.

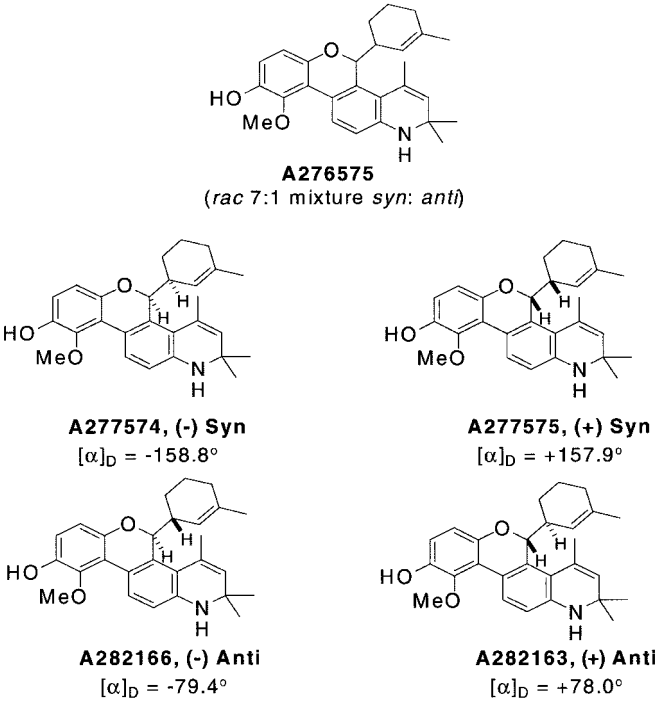

Fig. 1. Structure of A276575, It is composed of a 7:1 ratio of Syn- and Anti-diastereomers.

Experimental Procedures

Materials. [^{3}H]Dex (specific activity, 82–86 Ci/mmol) and [^{3}H]progesterone (Prog; specific activity, 97–102 Ci/mmol), EIA kit for PGE2 were purchased from Amersham Biosciences (Piscataway, NJ). Glass fiber type C multiscreen MAFC NOB plates were from Millipore (Burlington, MA). Hydroxyapatite Bio-Gel HTP gel was from Bio-Rad Laboratories (Hercules, CA). [^{3}H]Androstenedione and [^{3}H]thymidine were from PerkinElmer Life Sciences (Boston, MA). Histopaque-1077, Tris, EDTA, glycerol, dithiothreitol, protease–free bovine serum albumin, and sodium molybdate were obtained from Sigma-Aldrich (St. Louis, MO). Charcoal-stripped fetal bovine serum was from Hyclone (Logan, UT). Microscint-20 scintillation fluid was from Packard BioScience (Meriden, CT). Human skin fibroblasts (HSF) and human lung epithelial A549 cells were obtained from American Type Culture Collection (Manassas, VA). Primary HSF cells were from Clonetics (San Diego, CA). IL-1β was purchased from Roche Applied Science (Indianapolis, IN). Matched antibody pairs for human IL-6 were obtained from Endogen (Woburn, MA). Matched antibody pairs for human RANTES were from R & D Systems (Minneapolis, MN). All cell culture media and reagents were from Invitrogen (Carlsbad, CA). AmpliTaq Gold DNA polymerase was from Applied Biosystems (Foster City, CA). Synthesis and purification of the core of these compounds was performed as described previously (Coghlan et al., 2001).

GR and PR Radioligand Binding Assay. Cytosolic preparations of human GR-α isoform and human progesterone receptor (PR)-A isoform were prepared at Ligand Pharmaceuticals. Both receptor cDNAs were cloned into baculovirus expression vectors and expressed in insect Sf21 cells (Vegeto et al., 1992; Guido et al., 1996). Human GR-α and PR-A binding reactions were performed in Millipore Multiscreen plates. For GR binding assays, [^{3}H]Dex [~35,000 dpm (~0.9 nM)], GR cytosol (~35 μg of protein), test compounds, and binding buffer (10 mM Tris-HCl, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 20 mM sodium molybdate, pH 7.6 at 4°C) were mixed in a total volume of 200 μl and incubated at 4°C overnight in a plate shaker. Specific binding was defined as the difference between binding of [^{3}H]Dex in the absence and in the presence of 1 μM unlabeled Dex. For PR binding assays, [^{3}H]Prog [~36,000 dpm (~0.8

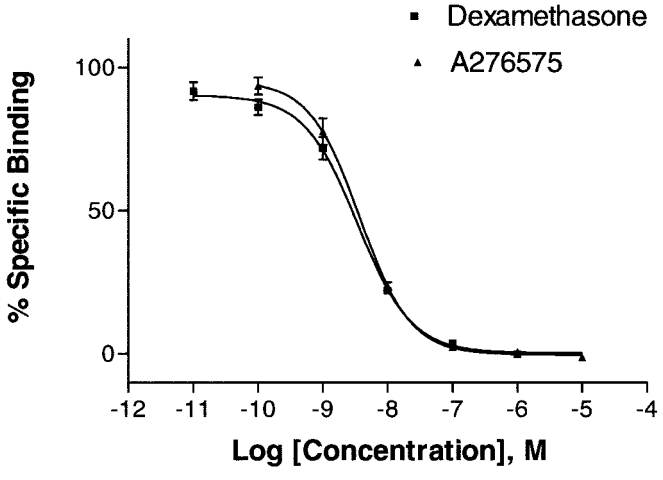

Fig. 2. Inhibition of [^{3}H]dexamethasone binding to cloned GR extracts by Dex and A276575. Experiments were conducted in duplicate as described under Experimental Procedures. Shown are the average values ± S.E.M. from three experiments. The Hill slope for Dex was −1.05 ± 0.11 (3), and −1.1 ± 0.11 (3) for A276575, indicative of binding to a single noncooperative population of GR.

TABLE 1

Binding affinity of A276575 and its four enantiomers for GR and PR

A276575 is a 7:1 mixture of Syn- and Anti-diastereomers. Values are average ± S.E.M. of the number of experiments in parentheses conducted in duplicate. For GR binding assay, total binding ranged from 1230 to 1430 dpm per well and nonspecific binding ranged from 62 to 85 dpm per well. For PR binding assay, total binding ranged from 1640 to 2640 dpm per well whereas nonspecific binding was 580 to 780 dpm/well. The range of test compounds was 10 pM to 10 μM.

Compound	K_i (*n*)		Ratio K_i
	GRα	PR-A	(PR/GR)
	nM	*nM*	
A276575	1.1 ± 0.1 (4)	18 ± 2.1 (4)	16
(+)-Syn enantiomer	13 ± 3.1 (3)	270 (2)	21
(−)-Syn enantiomer	1.2 ± 0.47 (4)	11.4 ± 3.5 (3)	9.5
(+)-Anti enantiomer	33 ± 7.2 (3)	670 (1)	20
(−)-Anti enantiomer	0.63 ± 0.13 (7)	52 ± 22 (4)	83
Dexamethasone	1.2 ± 0.22 (6)	8300 ± 1500 (6)	6900

nM)] and PR-A cytosol (~40 μg of protein) were used. Specific binding was defined as the difference between binding of [^{3}H]Prog in the absence and in the presence of 1 μM unlabeled Prog. The amount of protein used in binding assays was in the linear range of binding versus protein experiments. After an overnight incubation, 50 μl of hydroxyapatite (25%, w/v) slurry were added to each well and plates were incubated for 15 min at 4°C in a plate shaker. Plates were suctioned with a Millipore vacuum manifold and each well was rinsed with 300 μl of ice-cold binding buffer. Packard Microscint-20 (250 μl) was added to each well and shaken at room temperature for 20 min. The amount of radioactivity was determined with a Packard TopCount plate reader.

IC_{50} was determined from the Hill analysis of the binding curves. K_i of test compounds was determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973), $K_i = IC_{50}/(1 + [L^*]/[K_L])$, where L* is the concentration of radioligand, and K_L is the dissociation constant of the radioligand determined from saturation analysis. For GRα, K_L of [^{3}H]Dex was 1.1 nM, and [^{3}H]Prog was 4.5 nM. Competitive binding curves for Fig. 2 were analyzed by a nonlinear sigmoidal curve-fitting program by GradPad Prism Version 3 (San Diego, CA).

IL-6 Production in Nontransfected HSF Cells. HSF cells were cultured in 96-well plates to full confluence in DMEM plus 10% FCS and antibiotics. After removing the culture medium, test compounds prepared in 1.75% bovine serum albumin, DMEM, and antibiotics were added for 1 h at 37°C. IL-1β (1 ng/ml) was added to cells for overnight at 37°C, under 5% CO_2/95% O_2 atmosphere. Stock solutions of test compounds were prepared in 100% dimethyl sulfoxide and the final concentration of dimethyl sulfoxide in the assay was kept at 0.1%. Cell media were collected and the amount of IL-6 was determined by EIA protocols as described by Endogen.

MMTV-GRE Reporter Gene Cotransfection Assay. African green monkey CV-1 kidney cells were cultured in 12-well plates in DMEM containing 10% charcoal stripped FBS (Hyclone) and were transiently transfected using the calcium phosphate coprecipitation method (Truss et al., 1995). In brief, 5 μg/ml of human GR-expression plasmid vector (RSVhGR), 5 μg/ml of MMTV-LUC reporter plasmid, 2.5 μg/ml of pRS-β-galactosidase, and 7.5 μg of pGEM4 at a final concentration of 20 μg/ml were precipitated then added to cells. The MMTV-GRE promoter construct contains four GRE sequence, one NF2 sequence, and one OCT sequence. After 6 h, the media were changed to DMEM containing 5% charcoal-stripped FBS and test compounds for approximately 40 h. Cells were lysed in Triton X-100. Luciferase and β-galactosidase activity in cell lysate were measured. Cotransfection studies with the human PR-B, human mineralocorticoid receptor with the MMTV-LUC reporter were carried out in CV-1 cells as described above to determine cross-reactivity of compounds (Arriza et al., 1987; Berger et al., 1992).

Native Cell GRE Assay Based on Aromatase Activity in Nontransfected HSF Cells. The aromatase gene contains one GRE element in its promoter and was selected for the evaluation of GRE activities of compounds (Zhao et al., 1995). Primary HSF cells were cultured in DMEM containing 10% FBS in 48-well plates until confluence. Culture media were removed and replaced with 250 μl of fresh media containing test compounds for 24 h. Aromatase activity in cells were determined as described previously (Iida et al., 1990).

Con A-Induced Proliferation of Nontransfected Human PBMC. PBMC were isolated using procedures described previously (Wasik et al., 1990) and plated in 96-well plates at 50,000 cells per well. Test compounds and ConA (1 μg/ml) were added and cells were incubated at 37°C for 3 days. [^{3}H]Thymidine (0.5 Ci/well) was added to each well for 6 h. Cells were harvested onto a glass fiber mat, and after 24 h, radioactivity was determined with a Packard Matrix 9600.

PGE2 and RANTES Production in Nontransfected Human A549 Lung Epithelial Cells. A549 cells were grown to full confluence in F-6 medium containing 10% FCS and antibiotics. One day before compound treatment, cell medium was replaced with F-6 medium containing antibiotics but without FCS. Test compounds were added to cells for 40 min before IL-1β (1 ng/ml) addition. After an overnight incubation at 37°C under 95% O_2/5% CO_2, cell media were collected for determination of PGE2 and RANTES, a chemokine, by EIA procedures described by suppliers.

TABLE 2

Inhibitory potencies of A276575 and its four enantiomers on IL-1β–stimulated IL-6 production in nontransfected human skin fibroblasts

Values were the average ± S.E.M. of the number of experiments in parentheses conducted in duplicate. In the absence of IL-1β , the amount of IL-6 was between 19 and 118 pg/ml, and IL-1β (1 ng/ml) treatment, without test compounds, raised the levels to 4,173–49,000 pg/ml. The range of test compounds was 10 pM to 10 μM.

Compound	EC_{50} (*n*)	Dex Efficacy
	nM	%
A276575	10 ± 0.93 (8)	88 ± 3.5 (8)
(−)-Syn enantiomer	12 ± 3.0 (8)	90 ± 2.0 (8)
(+)-Syn enantiomer	84 ± 19 (7)	86 ± 2.2 (7)
(−)-Anti enantiomer	20 ± 6.5 (6)	97 ± 0.66 (6)
(+)-Anti enantiomer	180 ± 47 (5)	84 ± 2.4 (5)
Dexamethasone	1.4 ± 0.11 (5)	100 (5)

TABLE 3

Repression activities of A276575 and its four enantiomers on ConA-induced proliferation of nontransfected human PBMC

Values represent the average ± S.E.M. of the number of experiments in parentheses conducted in duplicate. The amount of [^{3}H]thymidine was 10 to 91 and 3,750 to 11,000 dpm/well in the absence and presence of ConA, respectively. The range of test compounds was between 10 pM and 10 μM.

Compound	EC_{50} (n)	Dex Efficacy
	nM	%
A276575	23 ± 15(4)	87 ± 2.7 (4)
(−)-Syn enantiomer	15 (2)	76 (2)
(+)-Syn enantiomer	180 ± 69 (3)	100 ± 4.6 (3)
(−)-Anti enantiomer	12 ± 3.6 (5)	93 ± 1.7 (5)
(+)-Anti enantiomer	450 ± 200 (3)	88 ± 1.9 (3)
Dexamethasone	1.5 ± 0.19 (5)	100 (5)

TABLE 4

Effects of dexamethasone, A276575, and its four enantiomers on MMTV-GRE promoter cotransfected with GR in CV-1 cells

Values represent the mean ± S.E.M. of the number of experiments in parentheses done in triplicate. The amount of normalized luciferase response is shown in the legend to Figure 3A. Compounds were tested between 1 pM and 10 μM.

Compound	MMTV-GRE Dex Efficacy
	%
A276575	1 (2)
(−)-Syn enantiomer	1 (2)
(+)-Syn enantiomer	1 (2)
(−)-Anti enantiomer	13 (4)
(+)-Anti enantiomer	1.5 (2)
Dexamethasone	100 (40)

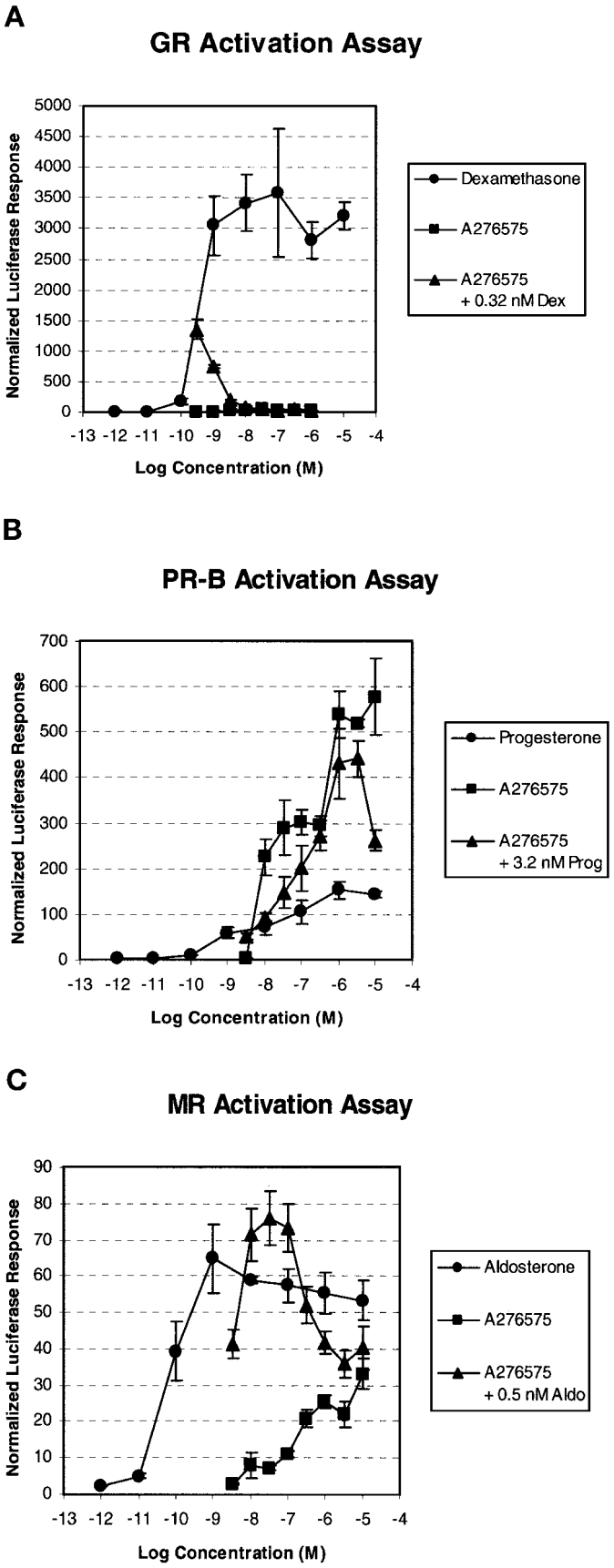

Fig. 3. A, A276575 is a GR antagonist at the MMTV-GR luciferase promoter. A-276575 did not stimulate MMTV-GRE construct but inhibited stimulation by Dex. The normalized luciferase response in presence of 1 pM and 10 nM Dex was 11 ± 9.5 and 3400 ± 460, respectively. Values were averages ± S.E.M. from three experiments conducted in triplicate. B, A276575 is a superagonist at the MMTV-PR-B luciferase promoter. Values were averages ± S.E.M. from three experiments conducted in triplicate. The normalized luciferase response in the presence of 1 pM and 1 μM progesterone was 3.8 ± 0.7 and 150 ± 19, respectively. C, A276575 is a partial agonist at the MMTV-MR luciferase promoter. Values were average ± S.E.M. from three experiments conducted in triplicate. The luciferase response in the presence of 1 pM and 10 nM aldosterone was 2.2 ± 0.2 and 59 ± 0.8, respectively.

Reverse Transcription Polymerase Chain Reaction Study in Nontransfected A549 Cells. Messenger RNA levels for RANTES and COX-2 were measured after A549 cells were treated for 24 h with IL1β alone and IL1β plus (−)-Anti- or (−)-Syn-stereoisomer of A276575. Total RNA was isolated by the TRIzol method (Invitrogen) and levels of RANTES and COX-2 messages were determined by RT-PCR. The primers used for COX-2 were: TTCAAATGAGATTGTGGGAAAATTGCT (bases 574–600, sense) and AGATCATCTCTGCCTGAGTATCTTT (bases 878–855, antisense) (Newton et al., 1997); the primers used for RANTES were: CCCTGCTGCTTTGCCTACAT (bases 120–139, sense) and CAAGAGCAAGCAGAAACAGG (bases 359–340, antisense). Cycling parameters were: denaturing, 94°C, 1 min; annealing, 54°C, 1 min; extension, 72°C, 1 min. Several aliquots were taken after 20 cycles to monitor the appearance of the COX-2 and RANTES messages.

Results

Affinities of A276575 and Its Four Enantiomers for GR and PR. A276575 showed high affinity (K_i = 1.1 nM) for GR and approximately 15-fold selectivity for GR over PR (K_i = 18 nM) (Table 1). Dex showed affinity similar to that of A276575 for GR and greater than 6000-fold selectivity for GR over PR. Of the four enantiomers of A276575, (−)-Syn and (−)-Anti enantiomers exhibited GR affinities similar to those of A276575 and 10- and 30-fold higher GR affinity than their corresponding (+)-enantiomers. The competition curves for both Dex and A276575 against [^{3}H]Dex binding showed Hill slopes near unity, consistent with binding to a single noncooperative population of GR (Fig. 2).

Repression Activities of A276575 and Its Four Enantiomers against IL-1β–Stimulated IL-6 Production in Nontransfected HSF Cells. Consistent with their high affinities for GR, A276575 and its (−)-Anti and (−)-Syn enantiomers repressed IL-1β–stimulated IL-6 production in HSF cells with high potencies (EC_{50}, 10–50 nM) and efficacies (>85% of Dex) (Table 2). Although (+)-Syn and (+)-Anti enantiomers of A276575 also repressed IL-6 production, they were ~10-fold less potent than their respective (−)-enantiomers.

Repression Activities of A276575 and Its Four Enantiomers against Con A-Induced Proliferation of PBMC. A276575 and its two (−)-enantiomers showed high potencies (EC_{50}, 15–25 nM) and efficacies (> 85% of Dex) in inhibiting Con A-induced proliferation of PBMCs (Table 3). Their potencies in inhibiting PBMC proliferation were approximately 10-fold weaker than that of Dex. The two (+)-enantiomers again showed approximately 10- to 30-fold lower potencies than their respective (−)-enantiomers.

MMTV-GRE Reporter Gene Assay in CV-1 Cells. In contrast to their high repression activities against IL-6 production and proliferation of PBMCs, A276575 and its four enantiomers exhibited minimal efficacies (< 5% of Dex) in activating the MMTV-GRE promoter in transfected CV-1

TABLE 5

Effects of Dexamethasone, A276575, and its four enantiomers on aromatase activity in nontransfected primary human skin fibroblasts

Values represent the mean ± S.E.M. of the number of experiments in parentheses conducted in duplicate. The amount of radioactivity incorporated into androgen in the presence and absence of Dex was 1190 to 1878 and 100 to 265 dpm/well, respectively.

Compound	Aromatase	
	EC_{50}	Dex Efficacy
	nM	%
A276575	13 ± 9	74 ± 19 (4)
(−)-Syn enantiomer	15 ± 5	80 ± 12* (3)
(+)-Syn enantiomer	180 ± 45	61 ± 16 (3)
(−)-Anti enantiomer	82 ± 35	82 ± 13 (4)
(+)-Anti enantiomer	1200 ± 610	56 ± 10* (3)
Dexamethasone	4 ± 1.1	100 (6)

* $P < 0.05$ versus Dex, unpaired Student&rquote;s *t* test.

cells (Table 4). In fact, A276575 antagonized Dex-induced GRE activation (Fig. 3A).

MMTV-PR-B and MMTV-MR Reporter Gene Assay in CV-1 Cells. In contrast to its antagonistic activity at the MMTV-GR reporter construct, A276575 behaved as a superagonist in the MMTV-PR-B luciferase assay (Fig. 3B), eliciting >300% of the response generated by progesterone. Its potency (EC_{50}, ~20 nM) at the PR-B construct was approximately the same as progesterone. On the MR-MMTV promoter, A276575 behaved as a partial agonist, exhibiting 50% of the response shown by aldosterone. Its potency was >100- to 1000-fold weaker than aldosterone (Fig. 3C).

Aromatase Activity in Nontrasfected Human Skin Fibroblasts. In contrast to their minimal activities in MMTV-GRE reporter gene construct, in primary HSF, A276575 and its four enantiomers showed significant but lower efficacies (56–81%) than Dex in the induction of aromatase expression (Table 5). Potencies of the (−)-Syn and (−)-Anti enantiomers were approximately 10- to 16-fold higher than their corresponding (+)-enantiomers. Therefore, A276575 induced greater GRE activation of aromatase than the MMTV-GRE reporter.

Differential Regulation of RANTES and PGE2 Production by (−)-Anti-and (−)-Syn-Enantiomers of A276575. Upon IL-1β challenge, the human A549 lung epithelial cells secrete a number of inflammatory mediators such as RANTES, PGE2, IL-8, and granulocyte macrophage-colony stimulating factor. GCs are known to suppress the production of these mediators (Newton et al., 1997). A276575 and its two (−)-enantiomers suppressed the production of PGE2 with potencies and efficacies similar to those of Dex (Table 6 and Fig. 4A). The two (+)-enantiomers of A276575 showed 5- to 10-fold lower potencies than their respective (−)-enantiomers. In contrast to their high PGE2 suppression activities, both A276575 (a 7:1 mixture of Syn- to Anti-diasteromers) and its (−)-Syn enantiomer were inactive against IL-1β–stimulated RANTES production in A549 cells. By contrast, the (−)-Anti enantiomer of A276575 showed high efficacy and potency in repressing RANTES production (Fig. 4B).

To confirm that the observed effects on RANTES and PGE2 production were caused by suppression of IL-1β–induced RANTES and COX-2 transcripts, we performed RT-PCR and assessed the effects of the two (−)-enantiomers. Figure 5 shows that IL-1β–induced RANTES was preferentially suppressed by the (−)-Anti enantiomer, whereas IL-1β–induced COX-2 message was equally suppressed by (−)-Anti and (−)-Syn enantiomers.

Discussion

Glucocorticoids are highly effective and widely prescribed anti-inflammatory agents. However, serious metabolic side effects associated with GC treatment have limited their use. It has been proposed that anti-inflammatory properties of GCs are attributable to inhibition of inflammatory mediators and metabolic side effects of GCs are related to GRE activation (Cato and Wade, 1996). GC ligands that maintain repression activity but have lower GRE activity may be safer than traditional steroidal GCs. Our efforts to develop such an agent led to A276575, an example of such a new class of nonsteroid GC ligand. A276575 has high affinity for GR and, similar to Dex, strongly represses the production of inflammatory mediators in several cellular systems, including IL-1β–stimulated IL-6 production in HSF cells, ConA-induced proliferation of PBMC, and IL-1β–stimulated PGE2 production in human A549 lung epithelial cells. In contrast, A276575 antagonizes Dex-induced activation of an MMTV-GRE reporter gene construct. However, compared with Dex, A276575 and its active enantiomers exhibit significant but lower induction of aromatase activity in nontransfected human skin fibroblasts. The efficacy of A276575 in the aromatase assay in HSF is much greater in its efficacy in the induction of the MMTV-GRE promoter construct. Possible explanations include differences of host cells, the transfected nature of the MMTV construct and the more complex transcriptional regulation of the aromatase gene.

A276575 contains two chiral centers and is a racemic mixture of 7:1 Syn- to Anti-diastereomers. To determine the stereospecificity of its interaction with GR, all four enantiomers were

TABLE 6

Effects of Dex, A276575 and its four enantiomers on IL-1β –induced PGE2 and RANTES production in nontransfected A549 lung epithelial cells

Values are average ± S.E.M. of the numbers of experiments in parentheses done in duplicate with <10% sample variation. The level of PGE2 was 13.6 ng/ml in the IL-1β (1 ng/ml) treated medium and was reduced to 0.6 ± 0.08 ng/ml by 1 μ M Dex (five experiments). The level of RANTES after IL-1β treatment was 112 ± 14 ng/ml and was reduced to 0.6 ± 0.08 ng/ml by Dex.

Compound	PGE2		RANTES	
	EC_{50}	Dex Efficacy	EC_{50}	Dex Efficacy
	nM	%	*nM*	%
A276575	3.7 ± 0.9	91 ± 4 (4)	N.A.[b]	15 ± 4 (4)*a*
(−)-Syn enantiomer	2.4 ± 0.69	96 ± 2.2 (5)	N.A.	17 ± 7 (5)
(+)-Syn enantiomer	26 ± 16	92 ± 3.8 (3)	N.A.	3 ± 1.6 (3)
(−)-Anti enantiomer	3.5 ± 1.1	98 ± 1.5 (5)	11 ± 3.2	78 ± 3.5 (3)
(+)-Anti enantiomer	140	85 (2)	N.A.	3 (2)
Dexamethasone	0.38 ± 0.07	100 (9)	1.9 ± 0.5	100 (9)

N.A., not applicable.

prepared and evaluated for GR affinity and activities in repression and GRE activation assays. As shown in Tables 1 to 3, the (−)-Anti and (−)-Syn enantiomers of A276575 exhibit high GR affinity and strong repression activity against IL-6 production in HSF, Con-A–induced proliferation of PBMC, and PGE2 production in A549 lung epithelial cells (Table 6). The two (−)-enantiomers also show similar potencies in activating aromatase activity in HSF and are devoid of any MMTV-GRE activity (Table 4). The (+)-enantiomers of A276575 are about 10-fold weaker in potency than their respective (−)-enantiomers. Surprisingly, the (−)-Anti enantiomer of A276575 is highly active against RANTES production in 549 cells, whereas the (−)-Syn enantiomers of A276575 and A276575 are inactive in repressing RANTES production (Table 6). Because PGE2 production is suppressed equally by (−)-Syn and (−)-Anti enantiomers of A276575, the inability of (−)-Syn enantiomer to repress RANTES production is unexpected. This result suggests that the (−)-Anti and (−)-Syn enantiomers of A276575 generate different conformations with the receptor. As shown by RT-PCR, the (−)-Anti enantiomer, not the (−)-Syn enantiomer, was able to suppress IL-1β–induced mRNA for RANTES. The promoter region of RANTES message contains a 12-*O*-tetradecanoylphorbol-13-acetate response element as well as several NF-κB binding sites, whereas COX-2 promoter contains only NF-κB sites. After binding to GC receptor, the (−)-Syn enantiomer may have a weaker interaction with the 12-*O*-tetradecanoylphorbol-13-acetate response element or NF-κB sites of RANTES promoter than the (−)-Anti enantiomer. The differences may also reflect differential interactions with corepressors and coactivators of RANTES and COX-2 message.

In summary, we have demonstrated that A276575 and its (−)-enantiomers have similar repression activities and lower GRE activities compared with Dex. The remarkable differences between enantiomers suggest that even subtle effects

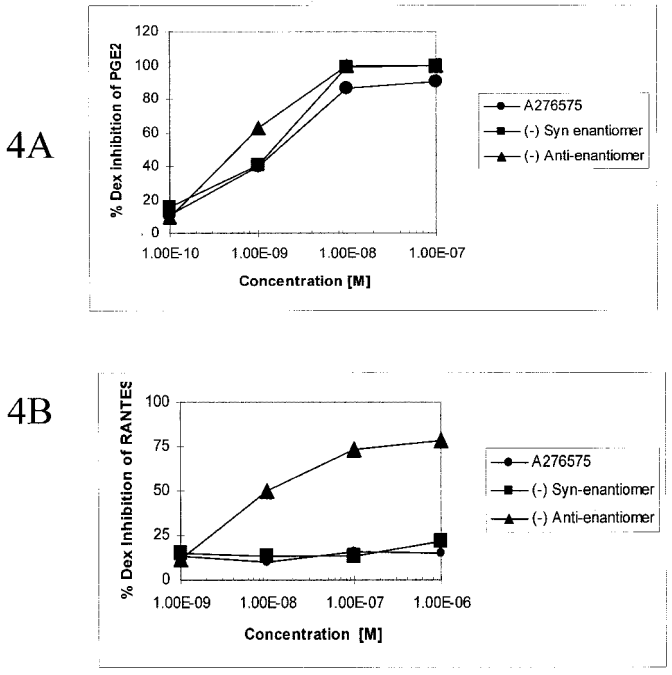

Fig. 4. Effects of A276575 and its (−)-Syn and (−)-Anti enantiomers on IL-1β (1 ng/ml)-induced PGE2 (top) and RANTES (bottom) levels in A594 human lung epithelial cells. Results shown were the average of at least two experiments conducted in duplicate.

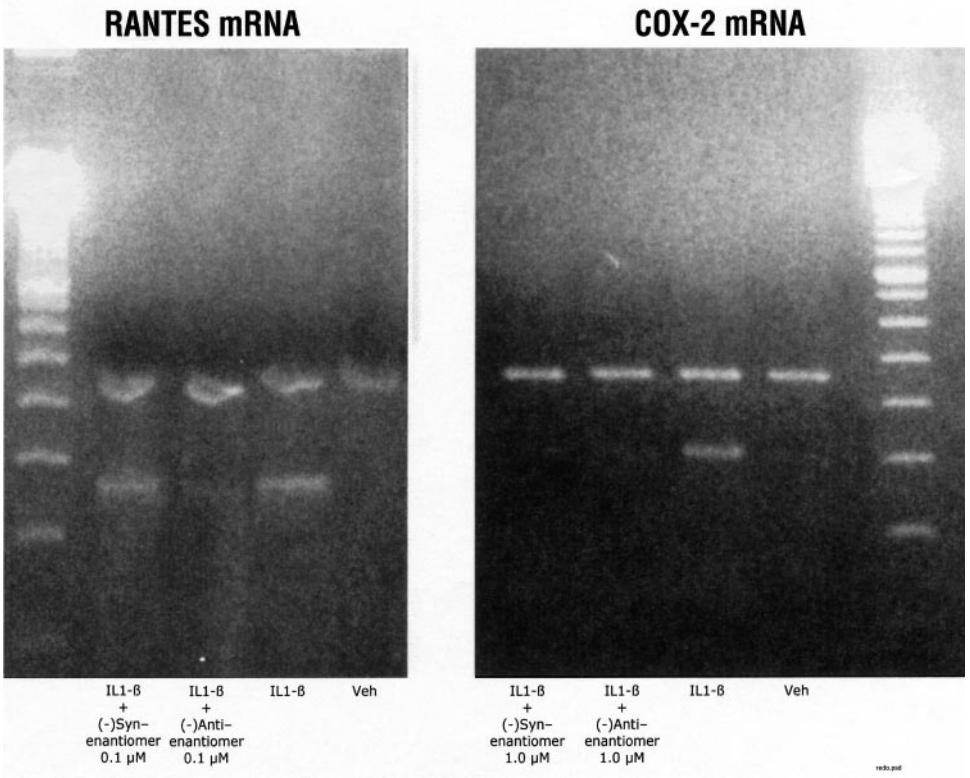

Fig. 5. Effects of (−)-Anti and (−)-Syn enantiomers of A276575 on IL-1β–induced COX-2 (left) and RANTES (right) mRNA in A594 cells. RT-PCR for COX-2 and RANTES were performed together with GAPDH as controls (450 bp). Both (−)-Syn and (−)-Anti enantiomers reduced COX-2 message, but only (−)-Anti enantiomer preferentially inhibited RANTES mRNA in A549 cells.

of ligand can impact receptor function. Given their in vitro profile, it is possible that these novel ligands possess good anti-inflammatory activities and lower metabolic side effects than traditional GCs. Because the (−)-Anti enantiomer is more effective than the (−)-Syn enantiomer in repression of RANTES, it is possible that the (−)-Anti enantiomer of this class of GR ligands will exhibit greater anti-inflammatory efficacy in vivo than the (−)-Syn enantiomer.

A-276575 exhibits high PR affinity and superagonist activity in the MMTV-PR-B transfection assays (Fig. 3B). These properties, which are undesirable and are not seen with dexamethasone, therefore limit its utility as an anti-inflammatory agent. Additional modifications of its structure led to analogs (e.g., compound 13) with high affinity for GR and low PR affinity and efficacy (Coghlan et al., 2001). Compound 13 also exhibits anti-inflammatory activity in Sephadex-induced eosinophil influx in rat lung (Coghlan et al., 2001).

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