Synthesis and Characterization of Non-Steroidal Ligands for the Glucocorticoid Receptor: Selective Quinoline Derivatives with Prednisolone-Equivalent Functional Activity

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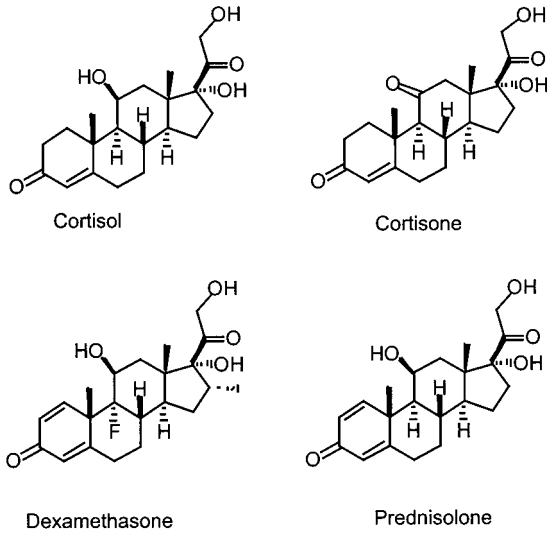
A novel class of functional ligands for the human glucocorticoid receptor is described. Substituents in the C-10 position of the tetracyclic core are essential for glucocorticoid receptor (GR) selectivity versus other steroid receptors. The C-5 position is derivatized with meta-substituted aromatic groups, resulting in analogues with a high affinity for GR (K_i = 2.4−9.3 nM) and functional activity comparable to prednisolone in reporter gene assays of glucocorticoid-mediated gene transcription. The biological activity of these novel quinolines was also prednisolone-equivalent in whole cell assays of glucocorticoid function, and compound **13** was similar to prednisolone (po ED_{50} = 2.8 mpk for **13** vs ED_{50} = 1.2 mpk for prednisolone) in a rodent model of asthma (sephadex-induced eosinophil influx).

Introduction

Glucocorticoids (GCs) have a pervasive role in human health and physiology. The endogenous members of this family, cortisol and cortisone (Chart 1), are involved in a broad spectrum of endocrine functions including metabolism of lipids, carbohydrates, and proteins, stress response, fluid and electrolyte balance, as well as maintenance of immunological, renal, and skeletal homeostasis.[1−5] Prompted by the isolation of GCs and identification of their structures in the mid 1930s, a monumental research effort in the 1940s and 1950s resulted in the discovery of newer, more potent analogues and an understanding of their biological properties, especially antiinflammatory effects. Prednisolone[6] and dexamethasone[7] emerged as benchmark drugs during this era. Although GCs are associated with a variety of clinical side effects, they are used for a breadth of antiinflammatory therapies while the search has continued for newer, more selective analogues. Despite considerable chemical and biological investigations, an understanding of the exact mechanism of action of these renowned molecules has only recently been uncovered. The discovery and cloning of intracellular receptors (IRs)[8−10] demonstrated that steroids act in association with specific members of this family. The glucocorticoid receptor (GR)[11] is a member of a rapidly growing family of intracellular steroid receptors[12] that regulate gene transcription. GCs act as the natural ligands for GR, and the resulting GR/ligand complex (GRC) regulates gene expression. Other steroid receptors such as estrogen (ER),[13] progesterone (PR),[14,15] mineralocorticoid (MR),[16] and androgen (AR)[17] also regulate transcription as complexes with their natural ligands, yet many of the natural and synthetic steroids bind to more than one member of this receptor family. This cross reactivity can result in undesired side effects: in the case of GCs, cross reactivity with MR, AR, and PR can be problematic.[18,19]

Chart 1



The GRC regulates gene transcription by two modes of action depicted in Figure 1.[20] When a steroid or other ligand L associates with GR in the cytosol, the resulting GRC is transported into the nucleus where it can regulate gene expression in either a monomeric or dimeric form. The antiinflammatory effects of GRC are believed to occur through the monomer, which adopts a conformation possessing an affinity for existing tran-

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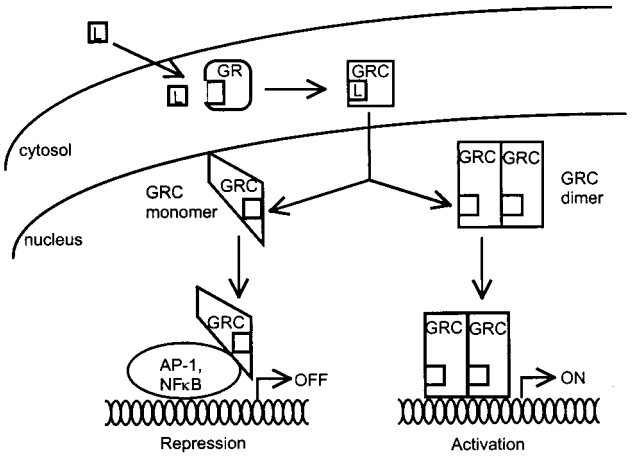

Figure 1. Differential functions of monomeric and dimeric glucocorticoid receptor/ligand complexes: repression and activation.

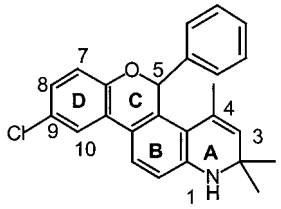

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Figure 2. Structure of lead dihydroquinoline **1**.

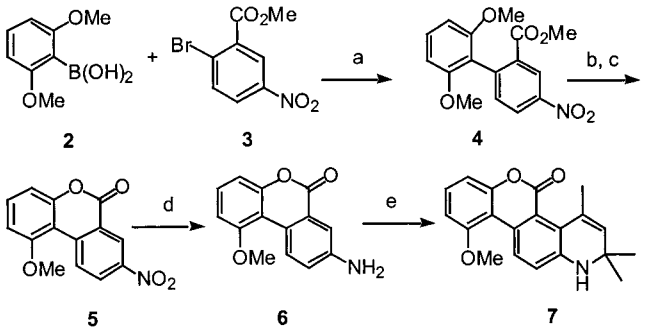
scription factors such as AP-1[21] or NFκB,[22,23] thereby repressing the transcription of proinflammatory cytokines and other inflammatory mediators.[24] The repression of proinflammatory agents via monomeric GRC is believed to be the primary mechanism for the antiinflammatory and immunosuppressive effects of GCs.[25] Dimeric GRCs behave as conventional steroid receptor/ligand transcription factors. These dimers adopt specific conformations that allow them to bind directly to particular DNA sequences called glucocorticoid response elements (GREs). When the GRC dimer binds to a GRE, gene activation occurs via stabilization of the transcriptional machinery at the start site of the promoter. GRC dimers are the endogenous transcription factors which associate with GREs, and most of the endogenous functions from transcriptional activation of GRC dimers are related to routine endocrine and metabolic processes.

To date there are no reported antiinflammatory, GR-specific nonsteroidal ligands that mimic GC function at the molecular level. We report the synthesis and biological characterization of novel, nonsteroidal GR-selective ligands whose functional activity is equivalent to prednisolone.

Chemistry

Our interest began with the disclosure of novel PR ligands which were cross-reactive with GR.[26] Quinoline analogues such as **1** (Figure 2) did not possess the desired degree of receptor selectivity. Moreover, they were inferior in assays of GR-mediated transcription when compared to reference standards such as dexamethasone or prednisolone.[26] However, we surmised that this novel tetracyclic core structure could be a general pharmacophore for the family of steroid receptors, and GR selectivity could be introduced in novel analogues. Once GR-selective analogues are prepared, functional equivalence to reference GCs could then be pursued in reporter gene assays of repression as well as whole cell models of GR function. Selective ligands with biological profiles comparable to those of commercial steroids could be tested in animal models of inflammation in comparison with clinically used GCs.

Scheme 1[a]



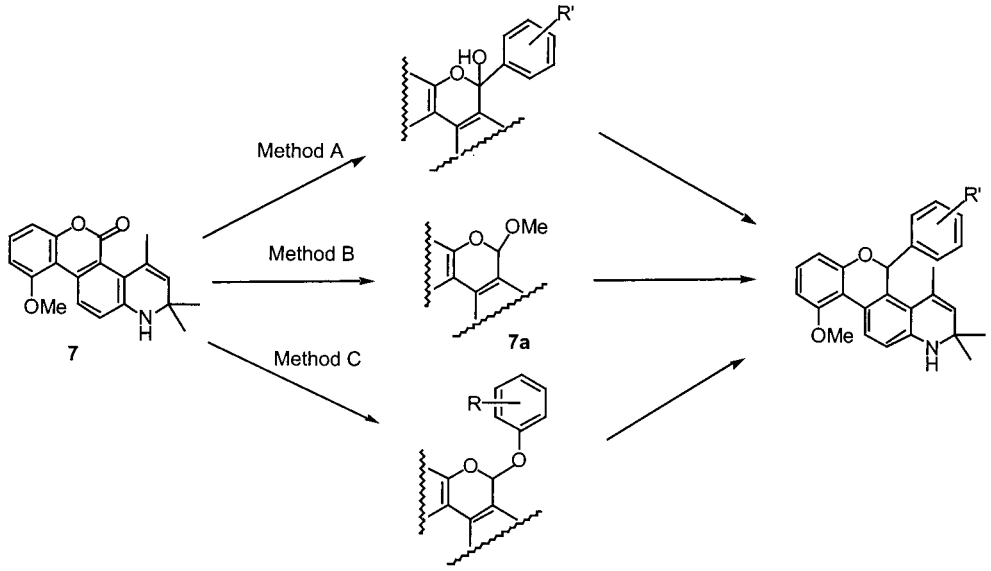
[a] Reagents: (a) $Pd(PPh_3)_2Cl_2$, Cs_2CO_3, DMF, 85 °C, 75%; (b) BBr_3, CH_2Cl_2, MeOH, 95%; (c) Cs_2CO_3, MeI, DMF, 99%; (d) H_2, 10% Pd/C, dioxane, 98%; (e) I_2, acetone, 105 °C, 45%.

Candidate GR ligands were prepared using a common sequence of general procedures amenable to inclusion of a variety of substituents in the D ring. A typical example of this route is shown in Schemes 1 and 2 for the C-10 methoxy analogues which began with the assembly of the tetracyclic quinoline skeleton. Core preparation and functionalization of the cores at C-5 was originally patterned after chemistry described earlier.[26] During the course of our efforts, we modified several of these procedures, particularly the introduction of the aryl group at C-5, which resulted in improved yields of analogous GR modulators. In the case of the C-10 methoxy tetracycle, the synthesis began with a modified Suzuki coupling of a known boronic acid with commercially available methyl 2-bromo-5-nitrobenzoate to deliver biphenyl compounds.

The electron-rich boronic acid **2** derived from resorcinol dimethyl ether[27,28] reacted with commercially available methyl 2-bromo-5-nitrobenzoate **3**[29] in the presence of dichlorobis(triphenylphosphine)palladium-(II) and cesium carbonate[30,31] to form the sterically hindered biphenyl ester **4** in high yield. Treatment of ester **4** with boron tribromide resulted in an efficient cleavage of the methoxy groups followed by spontaneous lactonization to the phenolic benzocoumarin, which was then alkylated with methyl iodide in the presence of cesium carbonate to provide the methyl ether **5**. The nitroarene functionality of ether **5** was reduced using catalytic hydrogenation to provide aniline **6** in high yield. The amino group of this benzocoumarin was then converted to the 1,2-dihydro-2,2,4-trimethylquinoline **7** by treatment with acetone and iodine via a modified Skraup ring annulation.[32−34]

With the tetracyclic benzocoumarin cores in hand, the task of functionalization at C-5 was undertaken. Three methods that were commonly used are shown in Scheme 2. Originally, method A involved organolithium addition to the C-5 carbonyl followed by reduction of the resulting hemiketal with triethysilane and BF_3 etherate or tri-

Scheme 2[a]



[a] Reagents for Method A: (i) ArLi, THF, −78 °C; (ii) Et_3SiH, $BF_3{\cdot}OEt_2$, CH_2Cl_2. Method B: (i) DIBAL, CH_2Cl_2; (ii) MeOH, H^+; (iii) ArMgX, $BF_3{\cdot}OEt_2$, CH_2Cl_2. Method C: (i) DIBAL, CH_2Cl_2; (ii) ArOH, $MgSO_4$; (iii) ArMgX, toluene.

fluoroacetic acid. Since this protocol gave satisfactory results with a limited number of core tetracycles, we turned to an alternative strategy involving a C-5 methyl acetal (method B) prepared by reduction of the C-5 lactone to the lactol followed by acetal formation under mild acid catalysis. The methyl acetals reacted smoothly with aryl Grignard reagents in the presence of Lewis acid catalysts such as BF_3 etherate. During this procedure, a deep green acetal/BF_3 complex develops at low temperature, and the reaction can be monitored by colorimetric titration during addition of solutions of arylmagnesium halides in ether to deliver the desired C-5 aryl analogues in high yields.

Although the acetal/BF_3 chemistry provided an assortment of derivatives, polar contaminants in the reaction such as small quantities of THF quenched the acetal/BF_3 complex, resulting in no desired product and recovery of starting acetal or lactol arising from hydrolysis. Method C was useful in those cases where the organometallic reagents were prepared in THF or where the polarity of the medium precluded methyl acetal/BF_3 complex formation. This method employs substituted phenoxy acetals, which are prepared with assorted phenols under mild acid catalysis. In many cases, these crystalline phenyl acetals were isolated and used without further purification. Phenyl acetals were treated directly with aryl organometallic reagents[35] to provide the desired C-5 aryl compounds. Overall, methods A, B, and C provided a breadth of candidate GR modulators for assessment in commendable yields.

Biology

Receptor Selectivity/Binding Assays. Assessment of the binding affinity for steroidal IRs was done following published procedures.[36] For GR binding assays, tritiated dexamethasone, human GR, and test compounds were incubated, and specific binding was determined as the difference between binding of [^{3}H]-dexamethasone in the absence and in the presence of 1 μM unlabeled dexamethasone. PR, MR, AR, and ER binding assays were similarly run using tritiated progesterone, aldosterone, testosterone, and estradiol, respectively.

Functional Activity. Although many compounds bind to GR, there are no reported nonsteroidal ligands for this receptor whose actions as repressors or activators of transcription are comparable with natural or synthetic glucocorticosteroids. We sought analogues equivalent in repression and activation to natural (cortisone, cortisol) or commercial (prednisolone) glucocorticoids. To assess the functional activity of candidate GR ligands, we employed a reporter gene cotransfection assay along with relevant assays of repression and activation in unaltered native cell lines. Cotransfection is routinely employed to characterize the interaction of a small molecule ligand of intracellular receptors. A pair of plasmids containing the IR of interest and a reporter gene construct is used to prepare transiently transfected mammalian cells that do not endogenously express the receptor under study. The plasmid encoded for the specified receptor is constitutively expressed, whereas the reporter plasmid is under the control of the same receptor-mediated promoter which lies upstream of cDNA for firefly luciferase, producing a quantifiable output upon receptor-mediated gene expression. Studies run in native cell lines were typically done for validation of the cotransfection data using unaltered cells which have been employed in steroid-mediated models of repression and activation.

Repression. The E-selectin assay is the cotransfection assay used to evaluate the repression activity of candidate GR ligands. This method uses a portion of the E-selectin promoter region that contains one copy of each of the AP-1 and NFκB sites. This segment of DNA has no known GREs, thereby reducing concerns about differentiating responses from repression versus activation mediated by monomeric or dimeric GRC. This construct is placed upstream of a luciferase gene expression vector in the HEP G2 cell line. Human GR is then transiently transfected into these cells, and

stimuli such as interleukin-1 (IL-1) or tumor necrosis factor (TNF) are used to induce expression of luciferase via the transfected E-selectin promoter construct. In the presence of repressors such as prednisolone or other functional GR ligands, expression of luciferase declines and this decrease is quantified. Internal standards such as prednisolone and dexamethasone are used to set a range of efficacy, and other commercial GCs are assayed for comparison with candidate GR ligands. Maximal efficacies of compounds are reported as a percentage of the maximal response observed for dexamethasone, and potency values are calculated as the concentration at half-maximal response for these curves.

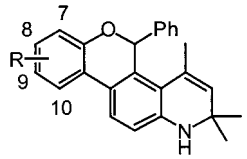
Although the E-selectin reporter gene assay provides a direct assessment of the functional activity of GR ligands, additional measures of repression in unaltered native cell lines with immunologically relevant endpoints were also followed. Glucocorticoids have been shown to inhibit the production of IL-1-induced IL-6 expression in human fibroblasts.[37] This effect has been shown to occur by way of a GR-mediated mechanism at the transcriptional level in native human cells, and conventional glucocorticoids effectively repress IL-6 production in this system.[38,39] Production of this cytokine is associated with proliferation of B and T cell lines, and circulating IL-6 levels increase in common inflammatory diseases such as rheumatoid arthritis.[40] Since gene expression associated with production of this cytokine represents another potential mechanism for the antiinflammatory effect of the steroidal ligands with GR, we employed IL-1-induced IL-6 expression in the native cell line to assess our novel GR ligands for functionality.

Activation. In the cotransfection assay for transcriptional activation via GREs,[41,42] the receptor plasmid containing hGR is introduced under the control of a constitutive promoter from the Rous sarcoma virus long terminal repeat. The reporter plasmid is a portion of the mouse mammary tumor virus (MMTV) promoter which lies upstream of a luciferase gene expression vector in CV-1 cells. The MMTV promoter contains four GRE sites, and the entire construct has no known affinity for AP-1 or NFκB. GR ligands will initiate luciferase gene expression using dimeric GRC interactions with these sites in the MMTV promoter. Maximal efficacy and potency values of compounds in this GRE activation assay are reported using dexamethasone as described for the repression assays above.

GR-mediated activation was also evaluated in unaltered human skin fibroblasts using standard assays employed for traditional glucocorticoids. Of the methods employed, measurement of the enzymatic activity of aromatase induced by GRE activation was selected. The aromatase assay is a biochemical method for measuring the enzymatic conversion of ^{3}H-androstenedione to estrogens. This process produces tritiated water as a byproduct which is then quantified using the same standards (dexamethasone and prednisolone) to determine maximal efficacy and potency.

In Vivo. Glucocorticoids greatly reduce lung eosinophil influx in asthma, and animal models that quantify this effect are commonly used as measures of effectiveness for this condition. We chose a sephadex-induced lung eosinophil influx in Brown Norway rats[43] to evaluate candidate GC ligands. In this experiment, sephadex beads are injected into the circulatory system, and the eosinophil influx into the lungs resulting from accumulation of the beads is measured by bronchoalveolar lavage (BAL). Reduction of eosinophil influx is assessed for candidate analogues in comparison with prednisolone, dexamethasone, and untreated controls.

Table 1. Receptor Binding Data: C-10 Substitution Improves GR Selectivity



		binding K_i (nM)[a]	
compd	R	GR	PR
1	9-Cl	9.3 ± 2.2	53 ± 11
8	8-OMe	1800[b]	–[c]
9	9-OMe	18 ± 3.9	390 ± 40
10	10-OMe	4.7 ± 0.70	–

[a] Mean values of at least three experiments done in triplicate with standard errors. [b] One experimental determination done in triplicate. [c] A hyphen indicates that the mean K_i values of at least three experiments were >5000 nM. Standard errors were not determined.

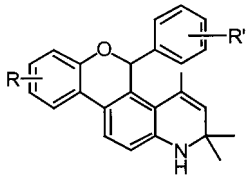
Results and Discussion

GR Selectivity. Since lead compound **1** also has an affinity for PR, we initially sought to optimize GR selectivity while minimizing PR cross reactivity. Installation of a substituent at C-10 of the core provided GR-selective analogues with very little affinity for PR. This trend is shown in Table 1 for the D ring methoxy analogues versus compound **1**. As the position of the methoxy group is moved around the D ring, GR binding and receptor selectivity are optimal for C-10 methoxy analogue **10**. Introduction of a variety of C-10 substituents suggests that this is a general trend for smaller substituents with the added benefit of improved activity on functional assays compared with prednisolone (see below). Binding assays with the remaining steroid receptors MR, AR, and ER (Table 2) demonstrated that this core modification provided GR-specific ligands with a breath of substituted phenyl groups at C-5.

Functional Ligands for the Glucocorticoid Receptor. Once GR-selective ligands had been prepared, we began to assess whether the resulting GRCs had favorable functional profiles. Our earliest investigations involved modification of substituents on the C-5 phenyl group on the GR-selective 10-methoxy core. We found that modification of the aryl substituents provided a remarkable number of active compounds. A representative group of these GR ligands is depicted in Tables 3 and 4. Modification of the aryl ring provided analogues that were prednisolone-equivalent in efficacy with comparable potency in in vitro models of repression and activation.

Modification at the meta position of the ring provided a group of functional GR ligands. Although prototype quinoline **1** was uniformly inactive in evaluation of GR-mediated function, compounds **12** and **13** are among the best of these ligands with a profile in reporter gene assays comparable to prednisolone.

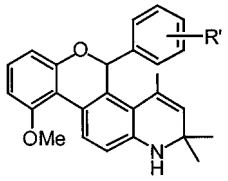
Table 2. Receptor Binding Data: Cross-Reactivity with Steroid Hormone Receptors[a]



			K_i (nM)				
compd	R	R′	GR	PR	MR	AR	ER[d]
1	9-Cl	H	9.3 ± 2.2	53 ± 11	1100 ± 57	1400 ± 6.4	‡
10	10-OMe	H	4.7 ± 0.70	–[b]	1682 ± 850	2660 ± 420	‡
11	10-OMe	3′-CF_3	11 ± 2.7	4000 ± 1300	493 ± 96	3360 ± 340	‡
12	10-OMe	3′-$OC(O)NMe_2$	2.4 ± 0.33	–	3914 ± 16	3066 ± 130	‡
13	10-OMe	3′-OCH_2SMe	4.0 ± 0.84	–	3838 ± 63	3115 ± 99	‡
prednisolone			2.4 ± 0.28	–	37 ± 12	2762[c]	‡

[a] Mean K_i values of at least three experiments done in triplicate with standard errors. [b] A hyphen indicates that the mean K_i values of at least three experiments were >5000 nM. Standard errors were not determined. [c] Mean values of two experiments done in triplicate with standard deviations. [d] ‡ indicates that the mean K_i values of at least three experiments were >1000 nM. Standard errors were not determined.

Table 3. Functional Repression of GR Ligands[a]



		cotransfection assay E-selectin		native cell assay IL-6	
compd	R′	potency (IC_{50}, nM) ± SEM	efficacy (% dex)	potency (IC_{50}, nM) ± SEM	efficacy (% dex)
prednisolone		2.1 ± 0.18	98	3.8 ± 0.16	95
10	H	123 ± 40	83	51 ± 0.19	62
11	3-CF_3	57 ± 33	93	32 ± 0.086	87
12	3-$OC(O)NMe_2$	6.0 ± 0.89	100	26 ± 0.14	92
13	3-OCH_2SMe	4.0 ± 0.89	100	16 ± 0.17	97
14	3-OCH_2OMe	23 ± 7.8	91	49 ± 0.12	77
15	3-Br-5-Me	18 ± 2.8	92	12 ± 0.061	91
16	3,5-di F	9 ± 2.8	96	11 ± 0.071	91
17	3,5-di Cl	67 ± 25	94	16 ± 0.11	94
18	3,5-di Me	18 ± 2.8	94	9.2 ± 0.061	90

[a] Mean values of at least three experiments done in triplicate with standard errors. All IC_{50} values were determined from full seven-point, half log concentration−response curves. Efficacies are shown using dexamethasone as the standard (100%). Standard errors for all efficacy values were = < 10%.

The presence of the C-5 stereogenic center in these selective GR ligands prompted the separation of two of the racemates, **10** and **17**, into their respective antipodes by HPLC. As shown in Table 5, the (−) enantiomers were equivalent to their corresponding racemates in GR binding and in vitro models of repression. The (−) compounds clearly showed superior efficacy and potency when compared to the (+) analogues, which were much less active in all assays examined. This biological preference for levorotatory isomers has also been observed for a closely analogous series of PR modulators where the (−) isomer was determined to have the '*S*' absolute stereochemistry.[44] By analogy, the (−) isomers in this series are also believed to have the '*S*' configuration.

We examined in vivo models of inflammation to determine whether the functional activity observed in vitro was suggestive of antiiflammatory activity in animal models of asthma such as the rat eosinophil influx assay. Several of the dihydroquinolines described above were tested and, of the examples described here,compound **13** was comparable to prednisolone (ED_{50} = 2.8 mpk for **13** vs 1.2 mpk for prednisolone).

Table 4. Functional Activation of GR Ligands[a]

	GRE cotransfection assay MMTV		native cell assay aromatase	
compd	potency (IC_{50},nM) ± SEM	efficacy (%dex)	potency (IC_{50},nM) ±SEM	efficacy (% dex)
prednisolone	8.0 ± 1.1	89	41 ± 6.0	85
10	300 ± 41	17	164 ± 86	18
11	44 ± 4.0	110	105 ± 1.1	52
12	12 ± 4.0	78	216 ± 63	74
13	9.0 ± 0.97	85	140 ± 19	102
14	28 ± 5.7	109	131 ± 16	30
15	29 ± 10	98	314 ± 168	19
16	10 ± 0.58	57	56 ± 27	90
17	107 ± 36	97	139 ± 29	82
18	24 ± 3.5	110	146 ± 41	45

[a] Mean values of at least three experiments done in triplicate with standard errors. All IC_{50} values were determined from full seven-point, half log concentration−response curves. Efficacies are shown using dexamethasone as the standard (100%). Standard errors for all efficacy values were = < 10%.

Table 5. Ligand Binding and Repression Data for Optical Isomers of GR Modulators[a]

		binding K_i (nM) ± SEM		native cell assay IL-6	
compd	sign of rotation	GR	PR	potency (IC_{50},nM) ± SEM	efficacy (% dex)
10	±	4.7 ± 0.70	–[b]	59 ± 0.19	62
19	+	240 ± 24	–	–	–
20	−	2.1 ± 0.40	–	30 ± 0.51	77
17	±	7.1 ± 3.2	–	16 ± 0.11	94
21	+	95 ± 4.9[c]	–	–	–
22	−	3.3 ± 0.53[c]	1560 ± 1100[c]	1.3 ± 0.076	70

[a] Mean values of at least three experiments done in triplicate with standard errors. All IC_{50} values were determined from full seven point, half log concentration−response curves. Efficacies are shown using dexamethasone as the standard (100%). Standard errors for all efficacy values shown were = < 10%. [b] A hyphen indicates that the mean K_i values of at least three experiments were >5000 nM, a functional potency that was not calculated due to low efficacy, or a functional efficacy <20%. Standard errors were not determined for hyphenated entries. [c] Mean values of two experiments done in triplicate with standard deviations.

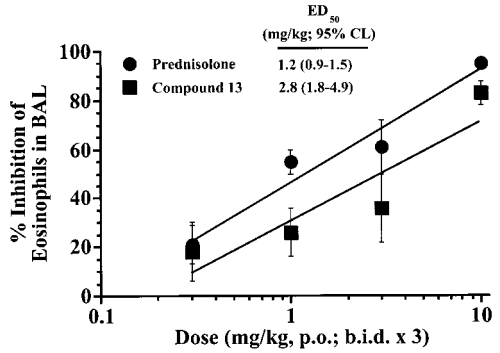


Figure 3. Comparison of **13** vs prednisolone in sephadex-induced eosinophil influx in rat lung.

Figure 3 depicts the dose response curves for prednisolone versus this early member of the new series of GR ligands. Compound **13** is fully efficacious in this assay with a dose response similar to that of conventional glucocorticoids.

Conclusion

A novel series of nonsteroidal, GR-selective ligands was discovered that mimic the functional effects of conventional glucocorticoids. The novel tetracyclic quinoline core represents a novel scaffold that provides functional ligands for the family of steroid receptors. Receptor binding as well as assays of activation and repression using cotransfected and native cell lines has enabled the characterization of a novel series of 10-substituted 5-aryl-1,2-dihydro-2,2,4-trimethyl-5*H*-chromeno-[3,4-*f*]quinolines that are comparable to prednisolone. The 10-substituent was the key for GR selectivity, and in vitro assays of these analogues demonstrated that meta-substituted C-5 phenyl analogues were comparable to GCs in GR-mediated function. Upon oral administration, compound **13** was comparable to prednisolone in a rodent in vivo model of asthma. To the best of our knowledge, this is the first report of nonsteroidal GR-selective ligands whose functional profile mimics those of natural glucocorticoids. This exciting result is currently under intense investigation aimed at separation of GR-mediated activation from repression. More comprehensive discussions of this SAR will be published in separate reports as well as other investigations of the in vitro and in vivo effects of this novel class of selective ligands for the glucocorticoid receptor.

Supporting Information Available: Detailed descriptions of reaction conditions, compound characterization data, and assay protocols are available free of charge via the Internet at http://acs.org.

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