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Design and evaluation of novel nonsteroidal dissociating glucocorticoid receptor ligands

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Abstract—A novel class of phenylpyrazole fused Wieland–Miescher ketone derivatives are high affinity, receptor specific, selective modulators of glucocorticoid receptor (GR) mediated transcription in vitro, dissociating transactivation, AP-1 repression, and NF-κB repression from each other.

Glucocorticoids bind to the glucocorticoid receptor (GR) in the cytosol whereupon the resulting GR/ligand complex translocates to the nucleus and associates with specific genomic glucocorticoid receptor response elements (GREs) to act as either an enhancer or represser of gene transcription. There are three general types of GREs: simple, composite, and tethering.[1] GR binds as a homodimer to simple GREs, usually an imperfect palindrome. Composite GREs are comprised of binding sites for GR as well as other transcription factors. At tethering GREs, GR modulates transcription indirectly through protein–protein interactions with other DNA-bound factors. Side effects associated with corticosteroid use include homeostatic disruption of routine endocrine and metabolic processes such as gluconeogenesis, bone metabolism, and electrolyte balance.[2] Simple GREs are present in the promoter region of genes responsible for regulating these essential functions.[3] In transgenic mice expressing a dimerization-deficient GR, GR mediated activation of genes with simple GREs (transactivation) is suppressed, but repression of gene expression via tethering GREs (transrepression) is intact and glucocorticoids act as anti-inflammatory agents as effectively as in wild-type animals. Thus, it has been postulated that molecules that preferentially act through tethering GREs may lead to anti-inflammatory drugs with reduced side effects.[4]

A survey of corticosteroids shows GR is tolerant to a variety of C and D ring substituents, whereas the A and B rings have been restricted to more subtle modifications such as incorporation of fluorines and unsaturation[5] (Fig. 1). This led us to choose the A and B rings as the core of our scaffold and to introduce substituents to mimic the C and D rings. The hydroxyl group of what would be the 11α position of cortisol was preserved as it has been shown to be important for selective binding of cortisol to GR versus PR and AR.[6] Results of an in silico screen using a homology model of GR and scaffold **1** suggested both phenyl and heteroaromatic substituents could be accommodated by the GR ligand binding pocket, leading to the synthesis of our first panel of compounds as shown in Scheme 1.[7] Commercially available racemic Wieland–Miescher (WM) ketone **2** was selectively thioketylated at the unsaturated carbonyl. Aldehyde **4** was obtained from homologation of **3** as a mixture of diastereomers in a ratio determined by ^{1}H NMR integration of aldehydic and alkenyl proton peaks to be 7:1. This ratio is in agreement with previous work,[8] where the equatorial aldehyde was determined to be the major product. Organometallic addition to **4**, followed by deprotection offered secondary alcohols **5**–**9** as mixtures of diastereomers ranging from 7:1 to 10:1 ratios for the single epimer at C-11 (**5**–**9** in Scheme 1).

Compounds **5**–**9** were then characterized in vitro. Binding of ligands to GR was determined using a flourescence polarization assay.[9] For transactivation assays, CV-1 cells were co-transfected with reporter gene TAT_3-DLO,[10] receptor plasmid pSG5-hGR,[11] and

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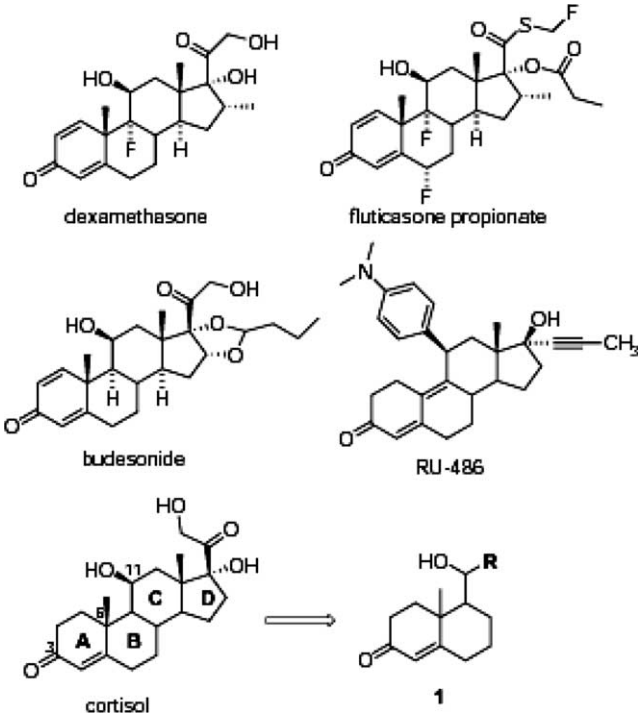
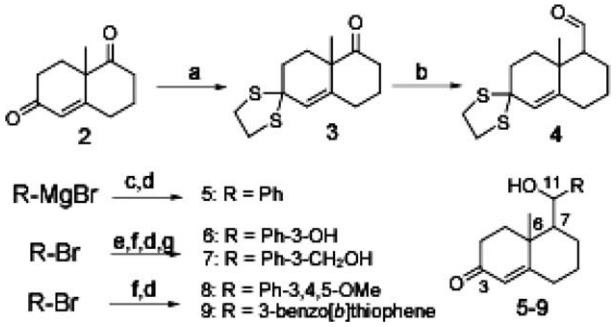


Figure 1. Synthetic and natural glucocorticoids.



Scheme 1. Reagents and conditions: (a) 1.1 equiv $HSCH_2CH_2SH$, cat. *p*-TsOH, AcOH, rt, 5 h, 82%; (b) (1) 4 equiv $Ph_3P^+CH_2OCH_3{\cdot}Cl^-$, 3.5 equiv KHMDS, THF; (2) 4 N HCl, MeOH, THF, 71%; (c) (1) **4**, THF, −78 °C → rt, 2 h; (2) H_2O, 0 °C, 93%; (d) 2.5 equiv $Hg(ClO_4)_2$, 4:3 MeOH/$CHCl_3$, rt, 5 min, 44–75%; (e) TIPS·Cl, imidazole, DMF, rt, 16 h, 80–90%; (f) (1) *t*-BuLi, Et_2O, −78 °C → rt, 3 h; (2) **4**, −30 °C, Et_2O, 0.5 h; (3) $NH_4^+Cl^-$, 20–80%; (g) 1.5 equiv TBAF, THF, −78 °C, 15 min, 65–85%.

CMV-βgal[12] as a transfection control. After transfection, cells were treated with either dexamethasone (DEX), or test compounds for 24 h and then assayed for luciferase activity. GR represses the activity of transcription factors AP-1[13] and NF-κB,[14] which are positive regulators of genes encoding proinflammatory cytokines and other inflammatory mediators. For our first set of compounds, K9 cells, an A549 cell line stably transfected with a κB response element upstream of a firefly luciferase gene[15] were stimulated with 5 ng/mL tumor necrosis factor—α(TNF-α) and incubated with either test compounds or dexamethasone for 16 h after which luciferase activity was assayed.

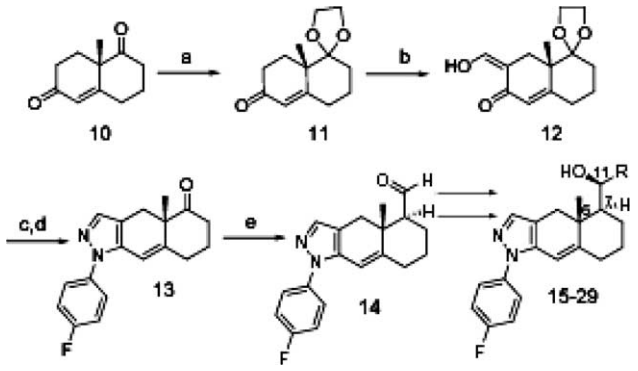
Binding and activity data for our first panel of compounds is shown in Table 1. Saturating responses for **5**–**9** could not be obtained due to cell death observed in reporter gene assays with ligand concentrations >10 μM. And while potencies could not be calculated, the activity of these compounds confirmed the predicted tolerance of the GR ligand binding pocket for C, D ring substitutions and demonstrated that dissociation between activation and repression (**6**, **9** vs **7**, **8**) can be modulated by varying the **R**. We then sought to modify the scaffold to improve affinity for GR without disturbing the functional significance of **R**. We chose to incorporate a *p*-fluorophenylpyrazole moiety fused to the A ring in our design.[16,17] Fusion of this heterocycle to the A ring of corticosteroids was discovered 40 years ago to enhance glucocorticoid activity in rats 30–2000 fold.[18,19]

The scaffold is synthesized in five steps as shown in Scheme 2.[20] Readily accessible enantiomerically pure (6*S*)-WM ketone[21] is first subjected to *trans*-ketalization with 2,2-methyl-ethyl dioxolane.[8] Literature procedures for the formylation of cortisol[18,22] and WM ketone derivatives[23] offered yields <50% while formylation with 2,2,2-trifluoroethylformate of the kinetically formed enolate[24] afforded reproducible yields of ~70%. Likewise, reports of arylpyrazole formation via condensation with *p*-fluorophenyl hydrazine from either formyl steroids[18,22] or formyl WM ketone derivatives[23] using either acetic acid or ethanol as solvents were either low yielding or unsuccessful. Refluxing benzene with azeotropic removal of water afforded greater than 80% yields. Deprotection under acidic conditions afforded ketone **13**, which was homologated to provide aldehyde **14** as

Table 1. Binding and transcriptional activity of ketones **5**–**9**

Compound	**R**	GR[a] IC_{50} (nM)	TAT[b] % DEX	NF-κB[c] % DEX
DEX	—	5 ± 2[d]	100	100
5	Ph	2100 ± 14	5 ± 2	54 ± 8
6	*m*-OH–Ph	ND	5 ± 1	53 ± 7
7	*m*-CH_2OH–Ph	ND	70 ± 10	14 ± 10
8	3,4,5-$(OMe)_3$–Ph	>2000	40 ± 15	14 ± 9
9	3-Benzo-[*b*]thiophene	165 ± 2	5 ± 2	74 ± 3

[a] GR binding was measured with a fluorescence polarization based competition assay using partially purified full length recombinant human GR-α.
[b] DEX (1 μM), or ligands **5**–**9** (10 μM). Values are expressed as percent luciferase activity relative to DEX.
[c] DEX (1 μM), or ligands **5**–**9** (10 μM). Values are expressed as percent repression of luciferase activity relative to DEX.
[d] All values are results of duplicate experiments performed in triplicate, error is reported as the standard deviation of the mean. ND = not determined.



Scheme 2. Reagents and conditions: (a) $HOCH_2CH_2OH$, *p*-TsOH, MED, 94%; (b) (1) LDA, Et_2O, −78 °C; (2) TFEF, (3) H_2SO_4, 70%; (c) 4-$FC_6H_4NHNH_2$·HCl, NaOAc, AcOH, benzene, reflux; (d) 1 N HCl, AcOH, THF, 81%; (e) (1) $Ph_3P^+CH_2OCH_3 \cdot Cl^-$, KHMDS, THF; (2) 4 N HCl, MeOH, THF, 70%.

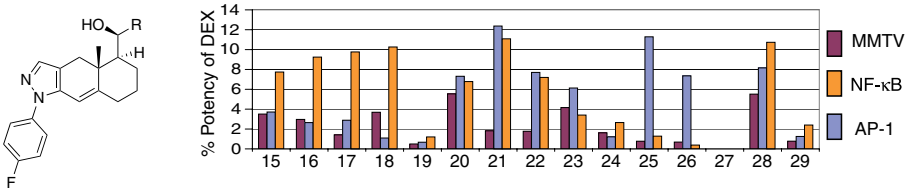
a 7:1 mixture of (6*R*,7*S*):(6*R*,7*R*) epimers, assuming diasteroselectivity of the reaction had not been affected by structural differences between **13** and **3**. Three subsequent recrystallizations from ethanol afforded 6*R*,7*S*-**14** in >90% enantiomeric excess (measured by NMR analysis of the chiral imine formed from the condensation of **14** and *S*(−)alpha-methylbenzylamine).

Test compounds were made by organometallic addition to the aldehyde as shown in Scheme 2, affording secondary alcohols **15**–**29** as a mixture of diastereomers (usually $\geqslant$ 9:1). Assuming our major epimeric product is (6*R*,7*S*,11*R*) based on Felkin–Ahn model predictions, initial studies towards optimizing the yield of this 'steroid-like' epimer showed that of organo-magnesium, -lithium, or -manganese reagents, the later in diethyl ether provided the highest diastereoselectivity. However, formation of organomanganese reagents was not always successful, and in these cases either Grignard or organolithium reagents were used instead. Either Grignard reagents or aryl halides were commercially available affording final compounds in one or two steps from scaffold.

In addition to the previously mentioned TAT assay, transactivation and transrepression were assayed in osteosarcoma cells(U2OS) stably expressing rat GR[25] using reporter plasmids $XG_{46}TL$[26] (activation), colA-luc[27] (AP1), and κB3-DLO[15] (NF-κB). In each case, CMV-βgal[12] was used as a transfection control. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was used to stimulate both AP-1 and NF-κB responses.

Affinity for GR and functional data from reporter gene assays for our second panel of compounds is shown in Table 2. GR binding of the compounds tested ranged from 10–200% of DEX. In functional assays, our compounds were ~20–100-fold less potent than DEX, but it is important to note that DEX is an extremely potent

Table 2. In vitro characterization of **15**–**29**



Compound	R	Binding	Activation				Repression			
		GR[a]	TAT		MMTV		AP-1		NF-κB	
		IC_{50} (nM)	EC_{50} (nM)	% DEX	EC_{50} (nM)	% DEX	EC_{50} (nM)	% DEX	EC_{50} (nM)	% DEX
DEX	—	1.4 ± 0.3	5.6 ± 0.8	100.0	0.2 ± 0.1	100	0.2 ± 0.1	100	0.3 ± 0.1	100
15	–$(CH_2)_7CH_3$	4.9 ± 3.8	—	41 ± 9	4 ± 1	116 ± 20	6 ± 0.9	68 ± 9	4 ± 1.4	49 ± 2
16	–$(CH_2)_3CH_3$	0.7 ± 0.2	—	46 ± 5	5 ± 2	79 ± 12	8 ± 2	84 ± 5	3 ± 0.3	85 ± 3
17	–(CH_2)–Ph	8.6 ± 5.0	—	26 ± 6	11 ± 3	82 ± 18	7 ± 3	72 ± 6	3 ± 0.8	61 ± 6
18	–*m*-CH_3O–Ph	2.3 ± 1.1	—	37 ± 10	4 ± 2	108 ± 16	19 ± 7	75 ± 8	3 ± 1.4	56 ± 7
19	–*p*-CF_3–Ph	14.0 ± 3.7	—	14 ± 4	31 ± 11	92 ± 4	31 ± 4	73 ± 11	24 ± 13	50 ± 3
20	–Ph	7.9 ± 1.1	—	24 ± 9	3 ± 0.7	99 ± 8	3 ± 0.6	73 ± 12	4 ± 1.2	63 ± 7
21	–*p*-F–Ph	9.2 ± 2.8	—	33 ± 10	8 ± 4	88 ± 11	1.7 ± 0.6	69 ± 10	3 ± 0.9	65 ± 7
22	–*p*-Cl–Ph	7.0 ± 2.0	—	49 ± 1	9 ± 0.6	134 ± 39	3 ± 0.2	80 ± 7	4 ± 3	68 ± 0.1
23	–*p*-CH_3Ph	2.5 ± 0.3	—	38 ± 2	4 ± 0.1	132 ± 28	3 ± 0.5	88 ± 5	8 ± 5	89 ± 2
24	–CH_2Ph	5.5 ± 1.1	—	26 ± 4	9 ± 0.9	130 ± 38	17 ± 1	77 ± 7	11 ± 4	75 ± 11
25	–3-Benzo-[*b*]thiophene	1.8 ± 0.3	—	44 ± 2	20 ± 4	112 ± 9	1.8 ± 0.4	71 ± 7	22 ± 9	67 ± 1.2
26	–2-Naphthalene	2.4 ± 0.6	—	39 ± 6	22 ± 5	127 ± 45	3 ± 0.9	71 ± 11	74 ± 35	54 ± 3
27	–*p*-(O–Ph)–Ph	15.5 ± 7.6	—	2.7 ± 0.3	—	1.1 ± 0.3	—	48 ± 20	—	28 ± 4
28	–*m*-OH–Ph	5.2 ± 2.4	—	26 ± 4	3 ± 0.2	133 ± 57	3 ± 1	112 ± 39	3 ± 0.9	68 ± 21
29	–*p*-OH–Ph	6.6 ± 0.7	—	12 ± 2	20 ± 1	115 ± 28	16 ± 3	77 ± 24	12 ± 7	51 ± 3

[a] In each experiment, full 7 log-point dose responses were obtained in triplicate. Error is represented as standard error of the mean of at least four separate experiments.

synthetic glucocorticoid. However, our compounds have comparable potencies to the natural glucocorticoid, cortisol (data not shown). For both AP-1 and NF-κB responses, the majority of the nonsteroidal compounds showed efficacies on the order of 50% or more of DEX, suggesting all, save **27** have the potential to exert significant anti-inflammatory effects in vivo.

Previous reports of nonsteroidal glucocorticoids demonstrate dissociation in vitro,[28–31] albeit between different cell types. Ours is the first to examine dissociation in a single cell type as shown in Table 2 (MMTV, AP-1, NF-κB, and inset chart). The EC_{50} of DEX was divided by that of each compound for each of the three assays. Comparison of the relative heights of the bars indicates degree of dissociation of the three responses relative to DEX. Following potency, this first panel of nonsteroidal arylpyrazole ligands can be placed into four distinct classes. Compounds **15**–**18** are slightly more effective in repressing NF-κB than AP-1 and GRE activation. Compounds **19**, **20**, **23**, **24**, **28**, and **29** show little dissociative activity. **21** and **22** are equipotent with respect to repression, and 5–7-fold selective for this activity over transactivation. **25** and **26**, however, are between 10–14-fold selective for repression of an AP-1 response over both GRE activation and NF-κB repression. Although many of the compounds tested were not as efficacious as DEX in NF-κB repression while acting as full agonists for activation, when compared to TAT activation in CV-1 cells, our compounds are both more potent and more efficacious in repressing AP-1 and NF-κB. This cell specific difference of transactivation has been reported for other gluocorticoids and is independent of the composition of the synthetic GRE.[32] Differences of expression levels of co-regulatory transcription factors and of GR itself between the two cells types likely contribute to the discrepancy of activation activity seen in our assays.

These compounds are also GR specific (data not shown). Binding to androgen receptor (AR) and progesterone receptor (PR) was measured with fluorescence polarization assays.[9] A radioligand whole cell binding assay was used to assess mineralocorticoid receptor (MR) binding.[33] In all cases selectivity for GR binding versus AR, PR, or MR is greater than two orders of magnitude.

The in vitro selectivity we have seen is on par with compounds that have proven dissociation in an in vivo model.[28,31] Clearly, with just this first panel of compounds, a significant range of mechanism specific transcriptional regulation has been observed, and it remains to be seen whether these compounds prove to be as effective and selective in vivo.

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