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Differentiation of in vitro transcriptional repression and activation profiles of selective glucocorticoid modulators

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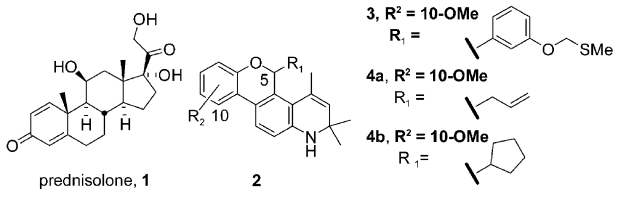
Abstract—The SAR at C-5 of the 10-methoxy-2,2,4-trimethylbenzopyrano[3,4-f]quinoline core leading to identification of (−) anti 1-methylcyclohexen-3-yl as the optimum substituent that imparts minimal GR mediated in vitro transcriptional activation while maintaining full transcriptional repression is described. The in vitro profile of these candidates in human cell assays relevant to the therapeutic window of glucocorticoid modulators is outlined.

Glucocorticoids (GCs) such as prednisolone (pred, **1**)[1] and dexamethasone[2] have long been considered some of the most effective antiinflammatory treatments for maladies ranging from asthma to rheumatoid arthritis. Chronic exposure to GCs, however produces a number of common, undesired effects associated with altered bone, glucose and lipid metabolism that manifests itself into GC induced osteoporosis, glucose intolerance, altered adipose differentiantion and fat redistribution (moon face, hump back).[3–6] Other hypertensive and cardiovascular side effects of commonly used GCs such as pred may be due at least in part to their cross reactivity with other steroid receptors such as the mineralocorticoid receptor (MR).

When GCs bind the cytoplasmic glucocorticoid receptor (GR), the resulting activated GR–ligand complex (GRC) translocates to the nucleus where it can either positively or negatively effect the expression of specific genes.[7–11] The up-regulation of genes requires a receptor homodimer to act directly as an endogenous transcription factor by binding to specific promoter regions termed glucocorticoid response elements (GREs). This activation function of the receptor complex has been associated with many of the undesired metabolic side effects described above.[4,12–14] The down-regulation of specific genes occurs by an indirect mechanism in which the activated GR complex binds to and inactivates other transcription factors essentially 'turning off' the genes they would normally regulate. The inhibitory action of the GRC on pro-inflammatory transcription factors such as AP-1 and NF-κB is believed to produce antiinflammatory effects by the repression of numerous cytokines, adhesion molecules and enzymes associated with synthesis of inflammatory mediators.[15-20] Among these are interleukin-1 (IL-1), TNF-α, GM-CSF, IL-3, IL-4, IL-5, IL-6, IL-8, E-selectin, I-CAM, Cox-2, iNOS, and PLA2. This repression mechanism is believed to be the basis of the antiinflammatory effect of glucocorticoid drugs.[21, 22]



We have been engaged in a research program dedicated to finding small molecule ligands of the glucocorticoid

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receptor with unique transcriptional repression/activation profiles that would provide efficacious antiinflammatory activity with diminished GC induced metabolic side effects. The 2,5-dihydro-2,2,4-trimethyl-1H-[1]-benzopyrano[3,4-*f*]quinoline core **2** is a non-steroidal tetracylic scaffold that has emerged as a general pharmacophore for several members of the nuclear hormone receptor family.[23–28] We have described our initial findings on the structure activity relationship of this core related to GR and found that proper C-10 substitution was critical for GR selectivity while C-5 substitution could be used to modulate transcriptional activity.[29,30] C-5 aromatic compounds such as **3** possess the full spectrum of transcriptional activation and repression activity equivalent to pred. C-5 non-aromatic compounds such as **4a** and **b** maintain efficacious repression activity in an E-Selectin (E-Sel) cotransfection assay while showing reduced levels of GRE activation in an MMTV-GRE cotransfection assay.[31] Furthermore, **4a** exhibited efficacy similar to pred in several in vivo models of inflammation, while displaying reduced side effects in at least two critical parameters (glucose metabolism and bone).[32]

Given the relationship between C-5 substitution and the transcriptional activity of these compounds, the prospect of further separating the antiinflammatory activity from side effects prompted a continued refinement of the C-5 position. Herein we describe the structure activity relationship study that led to the identification of 1- methylcyclohexen-3-yl as the optimum C-5 substitutent that imparts an improved in vitro transcriptional activation/repression profile. The biological profile of similar 9-OH-10-OMe substituted analogues has previously been reported.[33] We highlight here the in vitro profiles of the 9-H-10-OMe analogues in both cotransfection and native protein assays which we believe demonstrate potential for an improved therapeutic window.

Compounds were first evaluated in a competition binding assay to determine their intrinsic affinity for the α-isoform of the human glucocorticoid receptor (hGR). Mindful of progesterone receptor cross reactivity that has previously been noted in this series, progesterone receptor binding was also routinely monitored. Those compounds that demonstrated high affinity for hGR were then screened for their ability to functionally up- or down-regulate gene transcription in the MMTV-GRE and E-Sel co-transfection assays, respectively.[31] We have primarily utilized these co-transfection assays to guide our SAR studies to identify compounds worthy of more in depth analysis. Compounds of particular interest were then evaluated for their ability to activate or repress the transcription of genes relevant to the antiinflammatory or metabolic side effects of GC in human cell native protein assays. The repression of cytokine stimulated IL-6, collagenase and PGE-2 expression was used as a measure of antiinflammatory activity.[32,33] The activation of aromatase and tyrosine amino transferase (TAT) was used as a measure of endocrine related side effects while the repression of osteocalcin (OC) was used as a measure of effect on bone.[32]

Our strategy was to first rapidly survey the effects of substitution at each position of the allyl side chain in **4a** (Fig. 1a). Compounds **6** and **13**–**15** were prepared in a single step by Lewis acid promoted addition of known or commercially available allyl silanes to C-5 methyl acetal **5** (Fig. 2).[31] Palladium catalyzed methylation and carbonylation of vinyl bromide **6** and hydrolysis of allyl acetate **15** gave **7**, **8** and **16**, respectively (Scheme 1). Addition of 1-(*t*-butyldimethylsilyl)-1-methoxy ethene to **5** yielded the methyl acetate that was transformed to aldehyde **9** via Dibal-H reduction of the corresponding Weinreb amide. Terminally substituted allyl derivatives **10a**–**e** were prepared by Wittig olefination of aldehyde **9**. Treatment of **5** with methyl (triphenylphosphoranylidene)acetate gave the trans-methylbutenoate **11** that was hydrolyzed to provide allyl alcohol **16**. Hydride reduction of the corresponding mesylate provided the *trans*-butenyl analogue **12**.

Table 1 outlines receptor binding and cotransfection data for substituted allyl analogues. All of these derivatives exhibited potent GR binding affinity and were highly selective over PR. Small non-polar substituents at all positions maintained transcriptional activation and repression activity while more hydrophilic substituents at the 2′ or 3′ positions reduced both activation and repression potency and efficacy. Simple alkyl terminal olefin substitution demonstrated a distinct advantage by maintaining excellent E-selectin repression activity equivalent to the parent allyl compound **4a** while exhibiting a diminished ability to activate GRE. The 3′,3′-dimethyl analogue **10c** proved optimal with full E-sel repression function but significantly reduced ability to activate GRE (16% dex efficacy vs 68% for **4a**).

We next examined the effects of rigidification of the C-5 allyl group by its inclusion in a six-membered ring (Fig. 1b). Analogues **18**–**24** (Table 2) were prepared in a single step from **5** and known allylsilanes using the method described in Figure 2. An unattractive aspect of this tactic was the creation of a second stereogenic center in two of the three possible templates. Although these and related compounds were first prepared as mixtures of

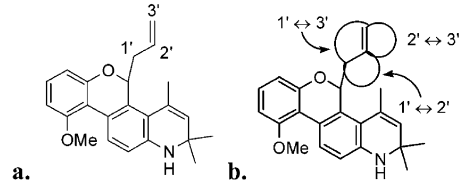


Figure 1. (a) Definition C-5 allyl substitution postions; (b) Three possible modes of cyclization C-5 allyl side chain.

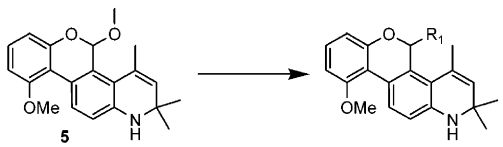
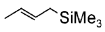
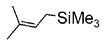
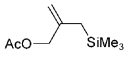
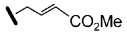
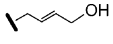


Figure 2. General synthetic method for additions of allyl silanes to methylacetal, **5**: allyl silane: CH_2Cl_2, $BF_3{\cdot}Et_2O$, −78 to 0 °C.

diastereomers, our strategy was to first analyze these mixtures in hope of identifying promising structural variants. We then planned to focus on these candidates and rigorously synthesize and characterize all stereoisomeric components.

Of the possible modes of cyclization (**18**–**20**), only the 1′3′ analogue **20** maintained E-selectin repression activity equivalent to **4a**. Interestingly, **20** also showed a nine-fold decrease in GRE activation efficacy compared to **4a**. Subsequent comparison of the five, six, seven and eight-membered cycloalken-3-yl rings (**20**–**23**) confirmed the six-membered ring to have the best balance of effective E-Sel repression and low GRE activation. Superimposing the optimum terminal olefin substitution (i.e., **10c**) with the C-5 cycloalkenyl **20** yielded 1-methylcyclohexen-3-yl analogue **24** that maintained excellent E-Sel repression and showed a further decrease in GRE activation (5% dex vs 68% dex for **4a**). We chose to concentrate on the 2:1 diasteromeric mixture **24** to determine the contributions made by each of its component isomers to its in vitro profile.

Table 1. In vitro receptor binding and cotransfection assay data for C-5 substituted allyl compounds. Compounds **6**, **13**–**15** were prepared according to the method described in Figure 2 utilizing the allylsilanes below. The phosphonium reagents used to prepare compounds **10a**–**e** are also provided

Compd	R_1	Reagent	GR binding (nM)	PR binding (nM)	GRE activation mean±SEM[a]		E-selectin repression[b] mean±SEM	
			mean±SEM	mean±SEM	IC_{50}, (nM)[c]	eff. (%dex)	IC_{50}, (nM)[c]	eff. (%dex)
Pred	—	NA	2.4±0.3*	—[d]	8.0±1.1*	89±19*	2.1±0.2*	99±1*
4a		NA	2.5±0.46*	1800±480	33±8.0 *	68±38*	13±6.3*	94±2*
13			2.2±1.1*	—	110±70	73±13	40±17	89±3
14			230±11	—	—	—	—	—
7		NA	3.1±0.7*	—	66±19	81±22	42±3.0	87±7
6			16±5.8*	—	240±68	54±6	110±37	74±2
8		NA	32±8.3	—	—	—	—	5±1
15			13±4.9*	—	390±40	48±16	160±1.3	74±6
16		NA	12±4.7*	—	310±6.6	50±1	141±60	80±9
10a		$EtPPh_3Br$	2.9±0.9	1200±983	39±20	46±11	23±6.4	91±4
12		NA	0.60±0.14	2100±1300	150±101	53±11	38±2.1	94±1
10b		*n*-$PrPPh_3Br$	3.1±1.3	—	89±15	24±2	88±54	81±2
10c		*i*-$PrPPh_3Br$	6.1±1.0*	1600±420	—	16±6	23±16	92±1
10e		$(cypent)PPh_3Br$	4.5±0.9	—	—	—	216.7	73±6
10d		$Ph_2P(O)CHF_2$	2.1±0.4*	2300±990	76±0.50	60±16	20±14	94±1
11		$Ph_3P=CHCO_2Me$	7.8±1.6*	—	220±48	46±10	87±10	77±2
17		NA	26±5.6*	—	—	7±1	150±8.9	44±28

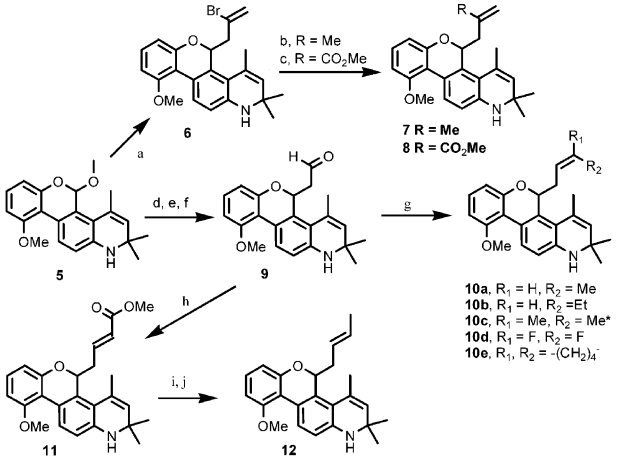
[a] Values with standard deviation represent the mean value of two experiments with triplicate determinations, values with an asterisk represent the mean value of at least three separate experiments in triplicate with standard error (SEM) and values without standard deviation represent a single experiment in triplicate.

[b] GRE activation efficacies are represented as the percentage of the maximal response of dexamethasone.

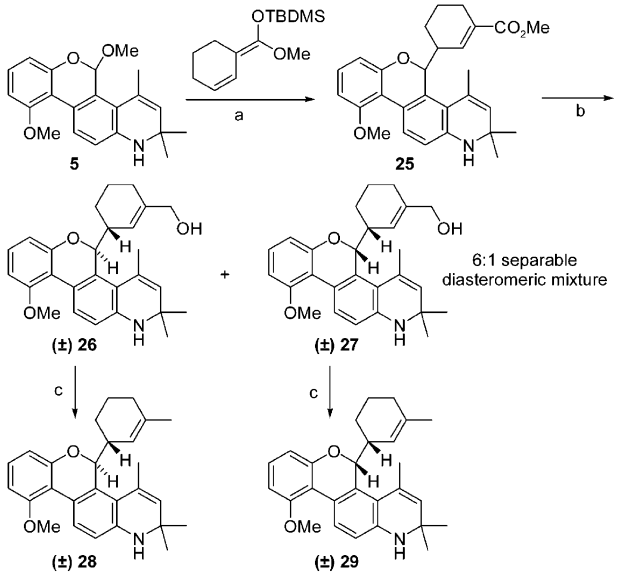
[c] All IC_{50} values were determined from full seven point, half-log concentration response curves in CV-1 cells and were calculated as the concentration at half the maximal response.

[d] A hyphen indicates a binding potency of >5000 nM, a functional potency that was not calculated due to low efficacy or not functionally efficacious. NA not applicable.

Unable to successfully separate the components of **24** directly by chiral HPLC, we resorted to independent synthesis of each diastereomer in racemic form (Scheme 2) with the hope of resolving them individually. Addition of 3-(*t*-butyldimethylsiloxy-methoxy-methylene)-cyclohexene to **5** followed by Dibal-H reduction of the resulting mixture provided a (6:1) separable mixture of allylic alcohol diastereomers (±) **26** and (±) **27**. These were each converted to methylcyclohexene analogues (±) **28** and (±) **29**, respectively, by reduction of their corresponding mesylates. The relative stereochemistry at the C-5 juncture of (±) **28** was unambiguously determined by single crystal X-ray crystallography to be *anti*. Both (±) **28** and (±) **29** were then easily resolved by chiral HPLC to provide their component enantiomers (**30**–**33**).[34] Consistent with previous results, the (−) enantiomers were far more active than their (+) antipodes.[24,31] The absolute stereochemistry of **30**–**33** was



Scheme 1. (a) (2-bromoallyl)trimethylsilane, BF_3-OEt_2, CH_2Cl_2, −78–0 °C, 90%; (b) Me_4Sn, $(PPh_3)_2PdCl_2$, HMPA, 85 °C, 60 h, 83%; (c) $(PPh_3)_2(CO)_2Ni$, Et_3N, MeOH, Δ, 16 h, 40%; (d) 1-TBDMSO-1-methoxyethene, BF_3-OEt_2, CH_2Cl_2, −78–0 °C, 90%; (e) NHMe(OMe)-HCl, Me_3Al, tol, 40 °C, 2 h, 62%; (f) Dibal-H, THF, −78 °C, 93%; (g) $RPPh_3Br$, *n*-BuLi, THF/Et_2O (5:2), −78 °C to rt, 25–90%; (h) $Ph_3PCHCO_2CH_3$, THF, 45 °C, 1 h, 80%; (i) $LiAlH_4$, $AlCl_3$, Et_2O, rt, 1 h, 53%; (j) $EtN(i\text{-}Pr)_2$, MsCl, CH_2Cl_2, −10 °C then $LiEt_3BH$, 38%.



Scheme 2. (a) BF_3–OEt_2, CH_2Cl_2, −25–5 °C, 73%; Dibal-H, THF, 0 °C, 86%; (c) $EtN(i\text{-}Pr)_2$, MsCl, CH_2Cl_2, −10 °C then $LiEt_3BH$, 73%, 74%.

Table 2. In vitro receptor binding and cotransfection assay data for compounds containing a constrained C-5 allyl group. Compounds were prepared according to the method in Figure 2 utilizing the allylsilanes below. Isolated reaction yields and diastereomeric product ratios based on ^1H NMR integration are provided where appropriate

Compd	R^1	Allylsilane	Yield	GR binding[a] (nM)	PR binding (nM)	GRE activation mean±SEM		E-selectin repression mean±SEM	
			Ratio (syn:anti)	mean±SEM	mean±SEM	IC_{50}, (nM)	eff. (%dex)	IC_{50}, (nM)	eff. (%dex)
Pred	—	NA	NA	2.4±0.3*	—	8.0±1.1*	89±19*	2.1±0.2*	99±1*
4a		$SiMe_3$	93%	2.5±0.46*	1800±480	33±8.0 *	68±38*	13±6.3*	94±2*
18		$SiMe_3$	85%	78±21	—	—	—	220	45±9
19		$SiMe_3$	45% (4:1)	11±3.7	—	—	13±1	320±29	49±16
20		$SiMe_3$	91% (1.1:1)	5.5±1.1*	2400±157*	13±3.3*	8±0.0*	21±1.2*	94±2*
21		$SiMe_3$	90% (1.5:1)	4.1±0.9*	2200±130*	12±4.9	34±1	12±4.2	96±1
22		$SiMe_3$	96% (1:1)	0.50±0.30	—	—	15±6	110±13	85±1
23		$SiMe_3$	73% (1.4:1)	65±15*	—	—	—	230	21±12
24		$SiMe_2Ph$	80% (2:1)	2.0±0.2*	980±86*	15±7.6*	5.0±1.0*	7.0±0.60*	97±1*

[a] Values for data in this Table are represented in identical fashion to those in Table 1.

Table 3. In vitro receptor binding and cotransfection assay data for pure 5-(1-methylcyclohexen-3-yl) enantiomers **30–33**

Compd		R_1	GR binding[a] (nM)	PR binding (nM)	GRE activation mean±SEM		E-selectin repression mean±SEM	
			mean±SEM	mean±SEM	IC_{50}, (nM)	eff. (%dex)	IC_{50}, (nM)	eff. (%dex)
Pred	NA	—	2.4±0.3*	—	8.0±1.1*	89±19*	2.1±0.2*	99±1.0*
(−) **4a**	(−)		1.4±0.62	1300±430	14±5.2	87±16	6.9	92±1.0
30	(−)-anti		0.7±0.1*	710±155*	40±0.6	84±20	19±0.4	90±6
31	(+)-anti		1700±752	—	—	—	—	2.0±2.0
32	(−)-syn		1.5±0.4*	430±96*	—	3.0±1.0	5.8±0.0	93±4.0
33	(+)-syn		49±37	—	—	16±4.0	440±91	49±18

[a] Values for data in this Table are represented in identical fashion to those in Table 1.

assigned by analogy to the parent allyl compound in which the active (−) enantiomer bears C-5 (S) configuration.[35]

Both (−)-anti **30** and (−)-syn **32** maintain high GR affinity, but show a two to three fold increased affinity for PR relative to other analogues in the C-5 alkyl series (Table 3). Given that the similar 9-hydroxy containing analogues were reported to possess PR 'superagonist' activity,[33] (−) **30** and (−) **32** were evaluated in the MMTV PR-B activation assay to determine their ability to functionally regulate PR. While (−) **30** was unable to activate PR in this assay, it exhibited weak, partial PR antagonism (241 nM, 61% antagonist of progesterone). (−) **32**, On the other hand, elicited a weak, partial PR agonism that is significantly lower than that seen with progesterone or either 9-hydroxy-10-methoxy-5-(methylcyclohexen-3-yl) stereoisomers reported previously (see Table 4 for comparison).

The transcriptional activity profile of (−) **30** in both E-sel and GRE cotransfection assays is quite similar to allyl (−) **4a**. Compound (−) **32**, on the other hand, is a full E-sel repressor, but shows a drastically reduced capacity to induce GRE activation [(−) **32** = 3% dex versus (−) **30** = 84% dex].

Table 4. PR binding and activation activity of (−) **30** and (−) **32** compared to their previously reported 9-OH counterparts[33]

			PR binding[a] (nM)	PR-B activation[b]	
			mean±SEM	IC_{50} (nM)	eff. (% prog)
30	(−)-anti	9-H	710±155*	—	13
	(−)-anti	9-OH	52±22	210	190
32	(−)-syn	9-H	430±96*	14	70
	(−)-syn	9-OH	11.4±3.5	7.1	260

[a] Values for data in this Table are represented in identical fashion to those in Table 1.
[b] PR-B activation efficacies are represented as the percentage of the maximal response of progesterone.

To determine the compounds transcriptional profile in a more physiologically relevant cellular context, we next evaluated (−) **30** and (−) **32** in a variety of human native cell assays evaluating gene products pertinent to both GC metabolic side effects and antiinflammatory properties. First, the repression of cytokine induced inflammatory mediators IL-6, PGE-2 and collagenase were used as a measure of potential antiinflammatory activity. While both diastereomers elicited nearly full repression of all of the inflammatory mediators (Table 5), (−) **32** was slightly more potent and efficacious than its counterpart (−) **30**.

The promoter regions of both aromatase and TAT are known to contain GREs and their activation has been associated with certain endocrine side effects of GCs. Consequently, the ability to up-regulate these proteins was used as a measure of potential metabolic side effects.[36,37] OC repression is considered a marker of the destructive osteoporotic effects of GCs and was evaluated as a measure of the compounds potential effect on bone.[37] Both (−) **30** and (−) **32** exhibit significantly reduced ability compared to pred to up-regulate aromatase and TAT (Table 5). In contrast to the GRE cotransfection assay, (−) **30** induces lower levels of GRE mediated transcription than (−) **32** with only 26% and 49% dex efficacy for aromatase and TAT, respectively. In addition, compound (−) **30** shows a much lower ability to repress OC compared to (−) **32** or pred. The combination of potent, efficacious repression of inflammatory mediators, the significantly reduced ability to negatively effect markers of GC induced endocrine and bone side effects and lack of PR agonist cross reactivity suggests (−) **30** to be the most attractive candidate to date. The efficacy and side effect profiles of (−) **30** in in vivo models of inflammation has not yet been studied.

From these studies, it is especially apparent that small, subtle structural changes in the GR ligand can produce significant differences in transcriptional profiles. We

Table 5. Human cell native protein assays for transcriptional activation of aromatase, TAT and transcriptional repression of osteocalcin, IL-6, PGE2 and collangenase for active enantiomers (−)-anti **30** and (−)-syn **32**

Compd	Aromatase[a]		TAT		Osteocalcin		IL-6		PGE2		Collagenase	
	EC_{50}, (nM)[c]	eff. (%dex)	EC_{50}, (nM)[c]	eff. (%dex)	EC_{50}, (nM)[c]	eff. (%dex)	EC_{50}, (nM)[c]	eff. (%dex)	EC_{50}, (nM)[c]	eff. (%dex)	EC_{50}, (nM)[c]	eff. (%dex)
pred	45±4.1	96±3.5	48±24	95±5.3	32	94	3.6±2.0	98±2.2	2.0±1.1	100±0.60	0.40±0.08	98 ±0.90
(−) **4a**	160±36	82±8.5	40±23	71±17	n.d.	n.d.	30±17	83±1.5	n.d.	n.d.	37±7.8	77±6.2
(−) **30**	640±35	26±6.8	110±31	49±3.6	—	38±7.0	62±31	82±1.3	35±14	95±3.4	32±7.6	89±4.0
(−) **32**	59±20	43±2.6	290±140	67±17	54±14	71±19	45±27	90±4.3	5.4±3.8	95±3.2	3.4±1.9	97±1.6

[a] Values for data in this Table are represented in identical fashion to those in Table 1. n.d. not determined.

have been able to achieve a significant in vitro differentiation of transcriptional repression/activation in human cell native protein assays that may be pertinent to the antiinflammatory and side effect profiles of GCs. Given that the moderate transcriptional differentiation seen with **4a** has been shown to translate to measurable in vivo improvements in at least two side effect parameters (glucose metabolism, bone), one would expect the more dissociated profile of (−) **30** to yield an improved in vivo therapeutic window.

Although the exact mechanism by which GR mediates transcription is not fully elucidated, one possible explanation for these differences are the varied abilities of the GRC to properly associate with accessory proteins necessary for normal function of the transcriptional machinery.[38] Ligand-dependent GC regulated transcription appears to require the association of the GRC with specific co-activator or co-repressor proteins.[39] These accessory proteins are recruited through a protein-protein surface interaction between an amphipathic α-helix on the coactivator/corepressor to a hydrophobic groove on the surface of the activated GRC. This hydrophobic binding groove is intimately associated with the ligand binding domain (LBD). The X-ray crystal structure of the ternary complex comprised of the GR LBD in its agonist form bound to dex and a coactivator motif from TIF-2 elegantly depicts this close association.[40,41] Accessory protein recruitment should therefore be highly dependent upon the ligand and the intricate conformational changes it imparts on GR upon binding. The ability of GR when bound to (−) **30** to differentially recruit known coactivators and co-repressors of GR has not yet been investigated.

References and notes

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