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5-Functionalized indazoles as glucocorticoid receptor agonists

Mei Bai [a], Grant Carr [a], Russell J. DeOrazio [a], Thomas D. Friedrich [b], Svetlana Dobritsa [a], Kevin Fitzpatrick [a], Peter R. Guzzo [a], Douglas B. Kitchen [a], Michael A. Lynch [a], Denise Peace [b], Mohammed Sajad [a], Alexander Usyatinsky [a], Mark A. Wolf [a,*]

[a] AMRI, 26 Corporate Circle, PO Box 15098, Albany, NY 12212-5098, USA
[b] Albany Medical College, 43 New Scotland Avenue, Albany, NY 12208, USA

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ABSTRACT

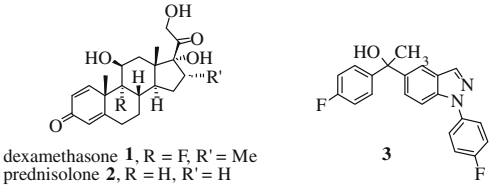
An indazole based series of glucocorticoid receptor agonists is reported. The SAR exploration of this scaffold yielded compounds with nanomolar affinity for the glucocorticoid receptor with indications of selectivity for the preferred transrepression mechanism; in vivo efficacy was observed in the mouse LPS induced TNFα model for compound **28**.

Glucocorticoid receptor (GR) agonists are highly effective anti-inflammatory drugs that have been used for more than 50 years. All of the marketed GR agonists are modified steroids. Orally administered GR agonists, such as dexamethasone (DEX, **1**) and prednisolone (PRED, **2**) (Fig. 1), are highly efficacious in combating inflammation; however, their use is tempered by the many side effects that all of these drugs induce.[1] The risk of developing one or more side effects such as osteoporosis, muscle wasting, skin thinning and others, increases with the higher doses of GR agonists and/or prolonged therapy.

Studies have revealed much about two GR mechanisms designated transactivation and transrepression.[2] It is widely proposed that the anti-inflammatory effects of glucocorticoid modulators are largely mediated through transrepression. In the transrepression mechanism, ligand bound GR interacts with other transcriptional factors, specifically AP-1 and NFκB, inhibiting their post-transcriptional activity. Many pro-inflammatory genes are regulated through the AP-1 and NFκB pathways, thus providing the underlying rationale of how transrepression is a key to the anti-inflammation properties of glucocorticoid modulators. Alternatively, it has been suggested that transactivation is responsible for many unwanted side effects. Thus, we sought non-steroidal glucocorticoid modulators which selectively act through the transrepression mechanism relative to the transactivation mechanism with the expectation that they may provide a superior drug therapy with reduced side effects for patients. There have been reports by several drug discovery groups of selective glucocorticoid agonists sometimes referred to as SEGRAs.[3]

A rational design approach was undertaken based upon known interactions from crystal structures of GR with steroidal ligands and the variety of non-steroidal ligands that had been reported. This approach led to the discovery of hit **3** shown in Figure 1. While only showing modest binding to the GR (IC_{50} = 2.1 μM), **3** provided a good starting point for further exploration. Similar structures were subsequently disclosed by another group[4] working in this area which has prompted us to disclose our results. To the best of our knowledge this is the first presentation of SAR data and biological activity for this scaffold.

The crystal structures of both agonist (PDB: 1p93) and antagonist (PDB: 1nhz) forms of the glucocorticoid receptor[5] allowed integration of a docking model into the discovery effort. A picture of **3** fitted to the modeled GR site is shown in Figure 2 below. A



dexamethasone **1**, R = F, R' = Me
prednisolone **2**, R = H, R' = H

3

Figure 1. Known steroidal GR agonists and initial hit molecule.

* Corresponding author. Tel.: +1 518 512 2348.
E-mail address: mark.wolf@amriglobal.com (M.A. Wolf).

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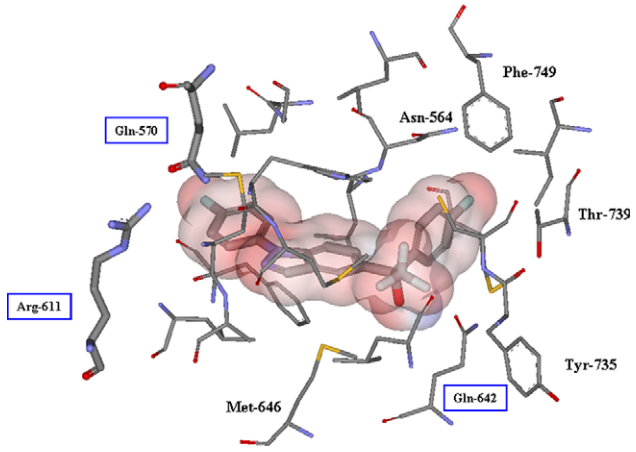
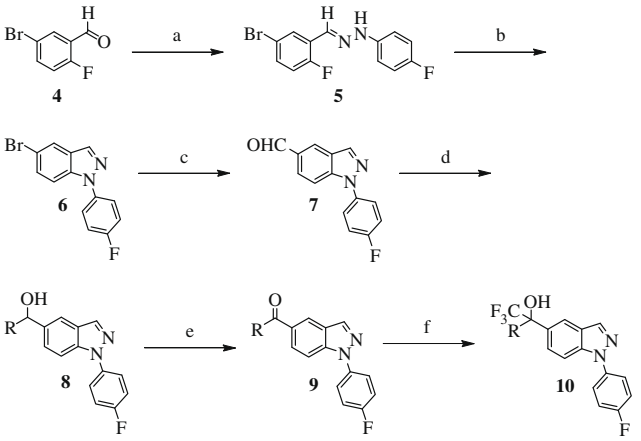


Figure 2. Model of initial hit **3** binding to GR.

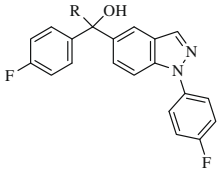
preference for the fluorophenyl moiety to be in close proximity with the Arg-611 and Gln-570 residues was observed. Close examination of the binding pocket shows a number of hydrophobic residues (Met-646, Phe-623, Met-601, Leu-732, etc.) closely encapsulating **3**. Figure 2 shows **3** with a van der Waals surface added to highlight its proximity to the surrounding amino acid residues. Using our docking model, the tertiary alcohol of **3** forms a hydrogen bond with Glu-642 (suggesting that this hydroxyl does not correspond to the secondary hydroxyl at C-11 of DEX, which has been shown to interact with Asn-564).[6] The docking model indicated that larger substituents off of the chiral carbon could take advantage of increased hydrophobic interactions.

The synthetic routes illustrated in Scheme 1 were used to prepare the indazole analogues.[7] Benzaldehyde **4** was reacted with 4-fluorophenylhydrazine to give hydrazone **5**. Hydrazone **5** was then cyclized to provide indazole **6**.[8] The versatility of 5-bromo-1-(4-fluorophenyl)-1*H*-indazole (**6**) allowed for the synthesis of numerous analogues in as few as 5–6 synthetic steps. Key intermediate (**7**) was prepared by palladium catalyzed carbonylation.[9] The addition of organometallic reagents (e.g., Grignard or organolithium) introduced the variable R group to provide secondary alcohols **8**. Following oxidation of **8** the trifluoromethyl moiety was introduced[10] to prepare the targeted tertiary substituted alcohols **10**. These analogues were first tested to determine their human GR



Scheme 1. Reagents and conditions: (a) 4-Fluorophenylhydrazine, diisopropylethylamine, CH_2Cl_2, rt, 16 h, 99%; (b) K_2CO_3, *N*-methylpyrrolidinone, 185 °C, 67%; (c) $Pd(Ph_3P)_4$, $NaCO_2H$, CO (250 psi), DMF, 110 °C, 37%; (d) RM THF, 0 °C, 1 h, 55–85%; (e) PCC, CH_2Cl_2, rt, 3 h, 55–90%; (f) (i) (trifluoromethyl)trimethylsilane, THF, rt, 3 h; (ii) TBAF, rt, 1 h, 35–77%.

Table 1
Structure–activity relationships at the 5-position



Compound	R	GR binding assay IC_{50} (nM) mean ± SD
11	H	>10,000
3	CH_3	2113
(+/−)-**12ab**	CF_3	161 ± 76
(−)-**12a**	CF_3	86 ± 43
(+)-**12b**	CF_3	>3000

binding affinity using a fluorescence polarization (FP) competitive binding assay.[11]

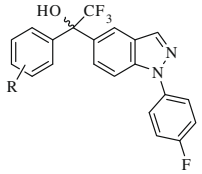
Varying substituents on the quaternary carbon at the 5-position led to profound changes in GR binding affinity. As can be seen in Table 1 below the introduction of the trifluoromethyl group (**12ab**) was extremely favorable for increasing binding affinity. The trifluoromethyl moiety has also proven beneficial for other GR ligands.[12] The separate enantiomers of **12ab** were isolated using chiral HPLC. Testing showed that there was a preference for one enantiomer, with the (−)-enantiomer (IC_{50} = 86 nM) being most active.

Variation in the 5-arylmethyl side-chain focused on the phenyl ring substituents. A subset of compounds illustrating the exemplary SAR at this position is found in Table 2 below. For ease of comparison, all compounds listed in Table 2 are racemic mixtures.

Following an optimization strategy using the Topliss operational scheme[13] (data not shown) it was discovered that 3-substituted phenyl rings generally demonstrated better GR binding affinity as illustrated by comparison of compounds **13**, **15**, and **20**. However, there were exceptions such as the chlorophenyl analogues (**16** vs **22**) where substitution at the 2-position was favored. 3,4-Disubstitution with halogens in the 4-position were tolerated (**14**, **18**, and **19**). Addition of a 4-fluoro substituent with 3-substitutions at least maintained affinity seen in the corresponding 3-monosubstituted analogues (**16** and **18**, **17** and **19**).

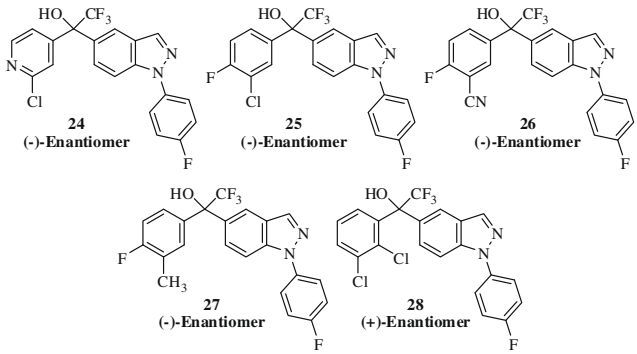
Docking experiments of a virtual library exploring this class of analogues indicated that compounds with 2-substituted aryl rings

Table 2
Structure–activity relationships at 5-arylmethyl position



Compound	R	GR binding assay IC_{50} (nM) mean ± SD
12	4-F	161 ± 76
13	4-Me	2089
14	3,4-Cl_2	178
15	3-Me	52
16	3-Cl	72 ± 57
17	3-CN	99
18	3-Cl-4-F	117
19	3-CN-4-F	30
20	2-Me	282
21	2-F	54
22	2-Cl	35
23	2,3-Cl_2	87 ± 11

Table 3
In vitro activity profile of leading compounds



Compound	GR affinity	Transrepression IL-6 assay		Transactivation (MMTV-Luc)	
	IC_{50} (nM) mean ± SD	EC_{50} (nM) mean ± SD	% DEX	50% Point (nM)	% DEX (conc)
24	14 ± 16	627 ± 256	60	NA	42% (3.3 μM)
25	49 ± 21	93 ± 21	85	ca. 1100	57% (3.3 μM)
26	54 ± 32	112 ± 37	90	NA	47% (3.3 μM)
27	59 ± 23	93 ± 3	87	NA	47% (3.3 μM)
28	34 ± 8	19 ± 4	87	ca. 350	70% (3.3 μM)
2	7 ± 4	3.0	94	55	99% (3.3 μM)

fit the receptor well. As a result both 2- and 2,3-disubstituted analogues were prepared and screened. The 2,3-dichlorophenyl analogue **23** demonstrated good binding affinity with an IC_{50} of 87 nM.

The most active racemates were separated to provide the individual enantiomers. The active enantiomers, as determined by the binding assay, were screened to determine their functional activity. Selectivity for transrepression over transactivation was examined using in vitro IL-6[14] and MMTV-luciferase (LUC)[15] assays. The agonist activity is reported as a percentage of the response induced by DEX. Prednisolone is included for comparison and the results are found in Table 3.

Initial results from the lead series showed that binding affinity for GR did not always translate into the desired functional activity (e.g., compound **24**, Table 3). An increased understanding of the SAR for the series drove improvements in activity seen in the transrepression (IL-6) assay (compounds **25–28**) and a better translation of binding affinity to functional IL-6 repression was observed. These compounds all achieve ⩾85% of the functional activity of DEX in the IL-6 assay and are less active in the MMTV-LUC assay, never reaching the same levels of activation seen with DEX or PRED, indicating enhanced selectivity for transrepression over transactivation versus current medications.

The five lead compounds (**24–28**) were profiled against a panel of nuclear hormone receptors including estrogen (ERα and ERβ, progesterone (PR-B), and androgen (testosterone) receptors.[16] All compounds showed >1000-fold selectivity for GR over these receptors. Additionally, these compounds were tested for off-target activity against 29 various receptors and ion channels.[17] No significant off-target activities (% inhibition <50% at 1 μM), including interaction with the hERG channel (IC_{50} >10 μM),[18] were noted for these compounds.

In order to demonstrate proof of concept for this series of nonsteroidal GR agonists, a mouse lipopolysaccharide (LPS) challenge model of inflammation was employed. It is known that LPS challenge induces an upregulation of pro-inflammatory cytokines such as TNFα in mice.[19] Known GR agonists, such as PRED, have been shown to down regulate this expression resulting in decreased inflammation.

Compound **28** possessed the most favorable in vitro profile of the lead compounds, including ADME assays (ex. metabolic stability, solubility, CYP inhibition, etc.) data not shown. The stability of compound **28** in acid was examined and the enantiomeric purity of compound **28** did not change upon exposure to 1 N HCl at 37 °C for 1 h; consequently it was selected for study in the inflammation LPS challenge model. Figure 3A shows that **28** demonstrated significant TNFα reduction relative to control at 3 mg/kg (25% reduction). The

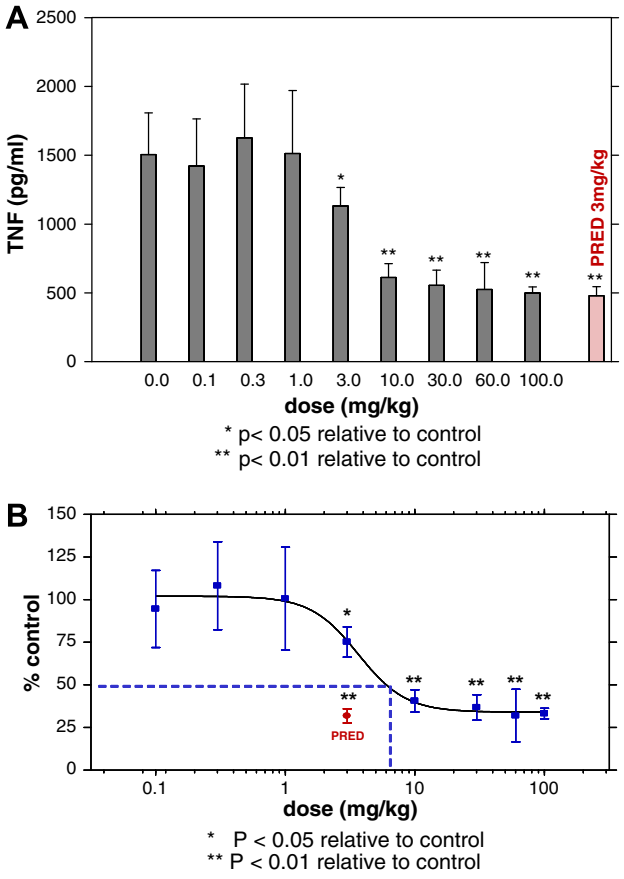


Figure 3. Compound **28** in LPS challenge model.

maximum reduction observed was 68% at 60 mg/kg. At 30 mg/kg it achieved similar results to that of PRED at 3 mg/kg. The dose response curve fitting of this data (Fig. 3B) shows that 50% inhibition of TNFα expression relative to control is obtained around 6 mg/kg. This experiment demonstrates that **28** is effective at suppressing inflammatory mediators in vivo.

In summary, (i) a rationally designed series has yielded compounds with nanomolar affinity for the glucocorticoid receptor with indications of selectivity for the preferred transrepression mechanism; and, (ii) in vivo efficacy was observed in the mouse LPS induced TNFα model for compound **28**. In addition to the positive in vitro and in vivo results, the profiling of these compounds against other nuclear hormone receptors, off-target receptors and ion channels shows excellent selectivity. The data indicates that these compounds represent an exciting new lead series for preparing selective glucocorticoid receptor agonists.

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14. Normal human dermal fibroblast cells (Cambrex, Cat. No. CC-2511) were cultured in Fibroblast Growth Medium 2 with BulletKit® supplements (Cambrex, Cat. No. CC-3132). Cells were plated on 384-well plates at 2500 cells per well in 50 μL of the growth medium and incubated at 37 °C with 5% CO_2 for 24 h. Test compounds (10 mM in 100% DMSO) were serially diluted at 100× assay concentrations in 100% DMSO and further diluted to 1.34× assay concentrations with Dulbecco's Modified Eagle's Medium (Fisher Scientific, Cat. No. SH3002202) supplemented with 1.75% bovine serum albumin (Sigma, Cat. No. A9647) and 1% penicillin-streptomycin solution (Fisher Scientific, Cat. No. SV30010). The growth medium in the cell plates was replaced with 30 μL of the assay medium containing test compounds, with samples done in quadruplicates. After cells were pre-treated with compounds for 1 h at 37 °C, 10 μL of 4 ng/mL IL-1β (Cytoshop, Cat. No. CYT-208) in assay medium was added to give a final IL-1β concentration of 1 ng/mL. Positive control wells received IL-1β but no test compounds and represented maximum (or 100%) production of IL-6. Background control wells did not receive IL-1βAdditional control wells received either 100 or 2 nM DEX as a standard treatment resulting in 100% or 50% inhibition of IL-6 production, respectively. The final DMSO concentration in all wells was 1%. Plates were incubated for an additional 24 h, and supernatants were harvested at the end of incubation and diluted eightfold with Dulbecco's Phosphate Buffered Saline (Fisher Scientific, Cat. No. SH3025602). IL-6 levels in the supernatants were determined with a Human Interleukin-6 Assay kit (Cisbio International, Cat. No. 62IL6PEB), according to manufacturer's instructions. IC_{50} values were determined using XLfit4.1 curve fitting software. A cell viability assay using Alamar Blue™ was duplexed with the IL-6 repression assay to eliminate cytotoxic compounds.
15. MDA-kb2 cells (ATCC, Cat. No. CRL-2713) were cultured in Leibovitz's L-15 medium with phenol red (Cellgro, Cat. No. 10-045-CV), supplemented with 10% Fetal Bovine Serum (FBS, Cellgro, Cat. No. 35-010-CV) and 2 mM L-glutamine (Cellgro, Cat. No. 25-005-C1), at 37 °C, without CO_2. Cells were plated on 384-well plates at 10,000 cells per well in 40 μL of L-15 medium without phenol red (GIBCO, Cat. No. 21083-027), supplemented with 10% FBS and 2 mM L-glutamine, and incubated at 37 °C without CO_2 for 5 h. Test compounds (10 mM in 100% DMSO) were serially diluted at 100× assay concentrations in 100% DMSO and further diluted to 5× assay concentrations with the above plating medium, supplemented with hydroxyflutamide (OHF, Toronto Research Chemicals, Cat. No. H942475), an antagonist of the androgen receptor also present in MDA-kb2 cells. 10 μL of 5× test compound/5 μM OHF mixtures were added to the cells. Negative control wells received no test compounds; positive control wells received 2 μM DEX. The final DMSO concentration in all wells was 1%; the final OHF concentration was 1 μM. Plates were incubated for an additional 20 h and then lysed with 30 μL of Luciferin lysis buffer containing 0.1 M Tris–HCl, pH 7.8, 2 mM $MgCl_2$, 0.6% Triton X-100 (Sigma, Cat. No. T9284), 10 mM DTT, 1 mM coenzyme A (Calbiochem, Cat. No. 234101), 0.3 mM ATP (Amersham, Cat. No. 27-1006-01) and 280 μg/mL luciferin (Sigma, Cat. No. L6882). Luminescence was measured in a CLIPR plate reader (Molecular Devices).
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