Tools for the Discovery and Characterization of GR Modulators:
A Comparison of Binding, Coregulator Interaction, and Transactivation Assays

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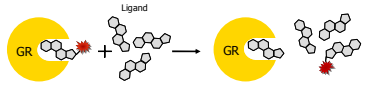
Introduction

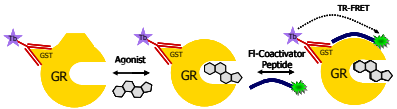
Glucocorticoids regulate a large number of physiological processes including inflammation, cell growth, bone density, metabolism and the cardiovascular system. Most of these effects are mediated by the glucocorticoid receptor (GR), making GR a central target for the development of drugs against pathologies in these areas. We have developed both cell-based and biochemical assays as tools to study GR pharmacology. These include competitive binding assays, coregulator recruitment assays and two different cell-based GR transactivation assays using a β-lactamase reporter gene readout: one uses a Gal4(DBD)-GR(LBD) chimera, while the other utilizes an MMTV promoter and endogenous GR. Using cell-based and biochemical assay tools, we have compared the binding affinities, coregulator recruitment, and transcriptional activation of a selected panel of compounds that contains glucocorticoids, GR antagonists and synthetic agonists.

Figure 1 – FP Competitive Binding Assay



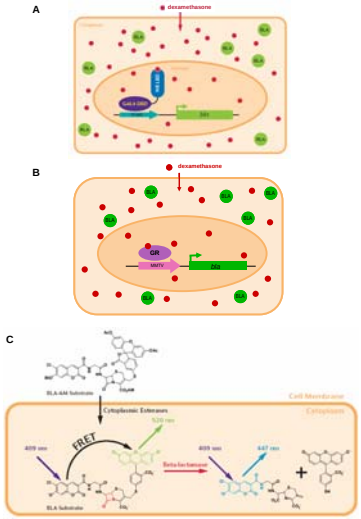
The PolarScreen™ GR competitive binding assay provides information on the **affinity of a compound for GR**, making it a great tool for screening a large library of compounds. In the assay, the receptor binds to the far-red fluorescently labeled tracer (or Fluormone™) in the absence of ligand, resulting in a high fluorescence polarization signal. Upon tracer displacement by ligand, the polarization signal is greatly reduced.

Figure 2 – TR-FRET GR Coactivator Recruitment Assay



The LanthaScreen™ GR coactivator recruitment assay assesses the **conformation of GR upon ligand binding.** Upon binding of agonist, helix 12 of the receptor undergoes a conformational change that results in an increased affinity for coactivator proteins. The receptor binding motif of the coactivators can be mimicked with fluorescein labeled peptides. Recruitment of these labeled peptides is detected by an increase in the TR-FRET signal between the Tb-anti-GST antibody (that binds to the GST tag of GR) and the fluorescein of the coactivator peptide.

Figure 3 – Full-length and Chimeric GR Cell-Based Assays



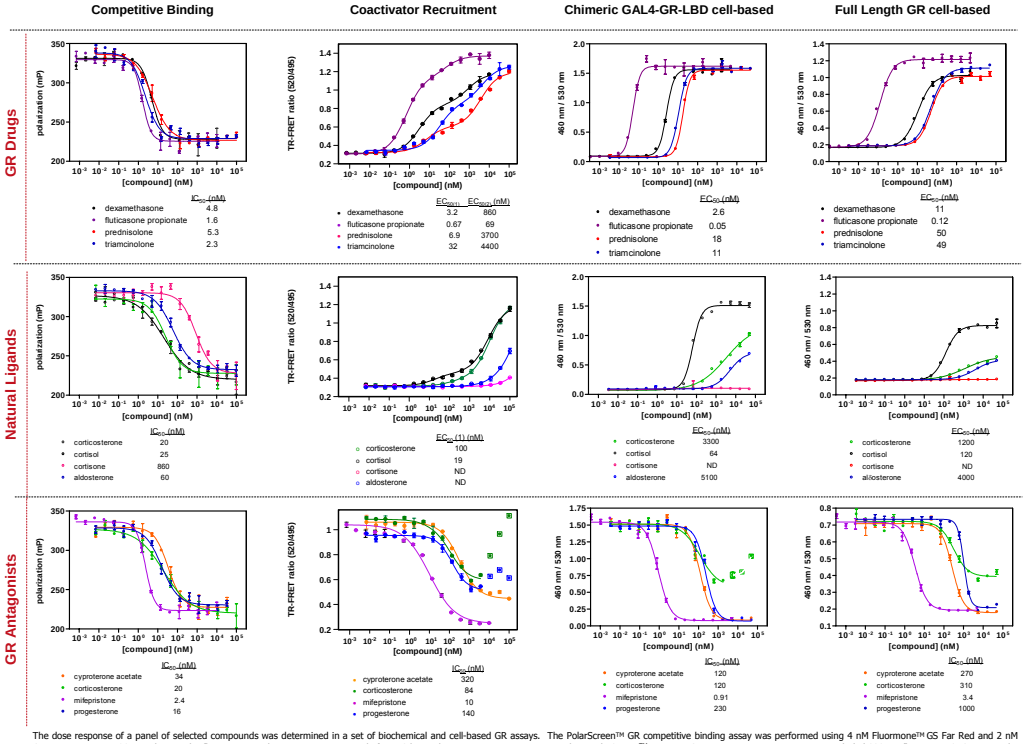
The GR cell-based assays report on the **transactivational activity of ligand bound GR. A.** GR-GAL4 cell based assay was developed by stably expressing a the GAL4-DNA binding domain fused to the GR ligand binding domain in HEK293T cells engineered with β-lactamase cDNA under transcriptional control of an Upstream Activator Sequence (UAS). **B.** The MMTV-*bla* cell based assay uses endogenous GR in HeLa cells that have been engineered to contain an MMTV promoter upstream of the β-lactamase gene. **C.** In both GeneBLAzer® assays, β-lactamase (BLA) expression is detected using a cell permeable FRET based substrate. In the cell, endogenous esterases de-esterify the substrate resulting in efficient FRET, which produces green fluorescence. Cleavage of the substrate by BLA results in the loss of FRET (blue fluorescence).

Table 1 – Comparison of Binding, Coactivator Recruitment, and Transactivation EC_{50} / IC_{50} Values for Selected Compounds

	6β-hydroxyprogesterone	β-estradiol	21-hydroxyprogesterone	2-methoxyestradiol	aldosterone	beclomethasone	beclomethasone 17-propionate	beclomethasone 21-propionate	beclomethasone dipropionate	betamethasone	budesonide	corticosterone	cortisone	cortisol	cortisone	cyproterone acetate	dexamethasone	fluticasone propionate	gestodene/ryvin	hydrocortisone 21-hemisuccinate	mifepristone	mometasone furoate	prednisolone	prednisone	pregnenolone	progesterone	promegestone	RU-24782	RU-24858	spironolactone	aldosterone	triamcinolone
GR-LBD binding assay	92	500	12	4400	60	3.1	1.3	49	3.9	4.9	2.5	25	20	25	800	34	4.8	1.6	ND	380	2.4	2.5	5.3	460	230	16	8.0	1.9	2.2	250	380	3.3
Coactivator recruitment assay Agonist mode	8500 p	ND	ND	ND	>10000	0.50	0.30	>10000	1200	0.40	0.30	>10000	100	0.73 p	ND	3500 p	0.30	0.20	ND	>1000 p	ND	0.30	0.6 p	ND	ND	3800 p	2000 p	0.65 p	0.5 sp	ND	ND	32
Gal4-GR-LBD cell-based assay Agonist mode	ND	ND	ND	ND	5100p	7.4	0.85	9.1	1.0	3.4	0.38	ND	3300p	64	ND	ND	2.6	0.05	ND	430	ND	0.09	18	ND	ND	ND	ND	41 p	44	ND	ND	11
Endogenous GR/MMTV cell-based assay Agonist mode	ND	ND	ND	ND	4000p	20	1.9	27	3.1	12	2.0	ND	1200p	130p	ND	ND	11	0.12	ND	1400	ND	0.12	50	ND	ND	ND	ND	160p	4800	ND	ND	49
Coactivator recruitment assay Antagonist mode	330 p	>10000	60 p	ND	800 p	ND	ND	420 p	20 p	ND	ND	310 p	50 p	ND	5000 p	300 p	ND	ND	ND	2000 p	14	ND	50	2000 p	800 p	100 p	30 p	10	10	3000	6500	ND
Gal4-GR-LBD cell-based assay Antagonist mode	260	3300	191	5700	650p	ND	ND	ND	ND	ND	ND	220	62 p	ND	3800	119	ND	ND	37	ND	0.91	ND	ND	3700	7600	234	148	6.5 p	ND	2300	3000	ND
Endogenous GR/MMTV cell-based assay Antagonist mode	410	4300	730	6800	4100 p	ND	ND	ND	ND	ND	ND	410	320p	ND	>10000	270	ND	ND	35	ND	3.4	ND	ND	6800	3700	1000	194	33 p	ND	3000	4700	ND

EC_{50} and IC_{50} values are in nM. p = partial response, ND = non detected

Figure 4 – Comparison of Dose Response Curves from Biochemical and Cell-Based GR Assays



The dose response of a panel of selected compounds was determined in a set of biochemical and cell-based GR assays. The PolarScreen™ GR competitive binding assay was performed using 4 nM Fluormone™ GS Far Red and 2 nM GR-LBD protein in a 20 µL volume. The fluorescence polarization was measured after a 3 hr incubation at room temperature. The LanthaScreen™ TR-FRET GR coactivator recruitment assay included 300 nM fluorescein-SRC1-4 peptide, 5 nM Tb-anti GST antibody and 1 nM GR-LBD protein in a 20 µL volume. The emission at 520 nm and 495 nm was measured after a 3 hr incubation at room temperature. For both cell-based assays, cells were incubated with compounds overnight and then loaded with LiveBLAzer™ - FRET B/G substrate loading solution in a final volume of 40 µL for 1.5 hrs before measuring the emission at 460 nm and 530 nm.

Conclusions

- Comparison of data in agonist and antagonist mode in Table 1 indicates that aldosterone, corticosterone, and RU-24782 behave as mixed agonists/antagonists.
- In the binding assay, all four of the GR drugs in Figure 4 show tight binding; however the coactivator recruitment assay and both cell based assays indicate that fluticasone propionate (Flonase®) is the most potent, followed by dexamethasone.
- Similar ligand affinity was observed for cortisol, corticosterone and aldosterone in the binding assay in Figure 4; however both cell based assays showed significantly greater potency for cortisol. Interestingly, cortisol and corticosterone demonstrated less difference in the coactivator recruitment assay.
- Although cortisone had significant binding with an IC_{50} of 860 nM in the binding assay, it showed little or no action in the coactivator recruitment and cell based assays.
- For the GR antagonists in Figure 4, mifepristone displayed the most potent IC_{50} in all of the assay formats.
- In summary, although the binding assay is a great tool to assess the affinity of a ligand for GR, the coactivator recruitment and cell-based assays provide additional complementary information that can be applied to the discovery and characterization of GR modulators.