AN ELECTROCHEMILUMINESCENCE-BASED LIGAND BINDING ASSAY FOR A G PROTEIN-COUPLED NEUROPEPTIDE RECEPTOR

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ABSTRACT

A non-radioactive, homogeneous and high throughput ligand binding assay was developed for a G protein-coupled neuropeptide receptor using Meso-Scale Discovery's electrochemiluminescence technology. The peptide ligand was labeled with $Ru(bpy)_3^{2+}$ at the N-terminus. The membrane receptors were immobilized on electrodes embedded in the assay plates. The binding of the ligand to the membrane receptor was detected via an electrochemically triggered light reaction. The Ru^{2+}-labeled peptide bound to the receptor in a saturable manner with a Kd at ~0.8 nM. This binding could be competitively displaced by a number of unlabeled peptide analogs. The pharmacological properties of these peptides determined by this non-radioactive assay were similar to those obtained from SPA assay using iodinated ligand. The overall performance of this assay in the HTS environment was equivalent or superior to the radioactive SPA assay, with an assay window consistently >10, a Z' factor > 0.7, and a throughput of 100 plates/day.

METHODS

Membrane preparation

HEK 293 cells expressing the GPCR of interest were resuspended in lysis buffer (50 mM Tris, pH 7.0, 2.5 mM EDTA, 0.5 mM PMSF) at 5 million cells/ml. Cells were homogenized with a Polytron and centrifuged at 1000g for 10 min at 4°C. Supernatants were centrifuged at 46,000g for 30 min. Pellets were resuspended in membrane buffer (50 mM Tris, 0.32 M sucrose, pH 7.0), aliquoted, and frozen in dry ice/ethanol and stored at -70°C until use.

Binding assay using Meso Scale technology

Membranes were diluted in assay buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) and dispensed in 1-2 µl (1 µg protein/well) in assay plates (MSD Multi-array™ High bind 384-well black, custom coated). The immobilization of the receptors to the bottom surfaces of the plates occurred when plates were incubated at room temperature for 1 hour. 13 µl of a blocking agent (MSD 2000-40003) was added to minimize non specific binding. Compounds were transferred, and then $Ru(bpy)_3^{2+}$-labeled peptide ligand was added to the assay plates. All of the above liquid handling procedures were performed using a 384-CybiWell. Plates were incubated at room temperature for 1 hour. Reading buffer containing tripropylamine was added using Multidrop and the ECL readings were immediately taken on Sector HTS reader. Total reaction volume was 25 µl/well. Final labeled ligand concentration was 0.5 nM.

Assay to determine color-Quenching effects

Since inhibition of binding is detected by a decrease in light intensity, color quenching by compound samples could result in false positive signals. To control for this effect, active samples were counter screened in an assay where biotinylated $Ru(bpy)_3^{2+}$-IgG was bound to the surface of avidin-coated plates.

Scintillation proximity assay (SPA)

Membranes were thawed and diluted in assay buffer (same as above). The following 4 reactants were added to assay plates (Packard 384-well white OptiPlate): 5 µg/well membranes, 0.2 mg/well SPA beads (Amersham RPNQ0001,), 0.2 nM 125I-ligand (Perkin Elmer), and compounds at desired concentrations. Plates were incubated at room temperature for 1 hour followed by a 2 minute centrifugation at 2000 rpm. TopCount 384 was used for scintillation counting (1 minute/well). Total reaction volume = 40 µl/well.
The assay was slightly modified for LeadSeeker readouts as follows: 1 µg/well membranes and 0.2 mg/well SPA beads (Amersham RPNQ0260). Plates were imaged for fluorescent intensity using LeadSeeker for 5 minutes. Total reaction volume = 20 µl/well.

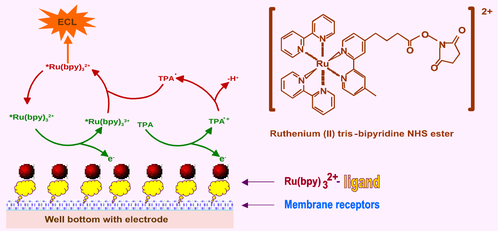
FLIPR assay

Cell culture: An HEK 293 cell line expressing the GPCR receptor of interest was cultured in DMEM with 10% FBS, 1% penn Strep, 1% L-glutamin, 500 µg/ml G418, and 200 µg/ml hygromycin. Cells were plated (Poly-D-Lysine coated, BD Biocoat #356663) the day before experiment at the density of 25000 cells/well (20 µl/well) in the same medium.

FLIPR assay: Cell medium was replaced by 20 µl/well of 1 X non-wash calcium indicator dye (Molecular Devices #R8033) in assay buffer (10% Hanks, 50 mM HEPES, and 0.1% BSA). After 1 hour incubation at 37°C, peptide agonists were applied to cell plates in the FLIPR and a series of images were taken subsequently for 2 minutes. The extent of GPCR activation was measured by the Max-Min response in the fluorescence signal.

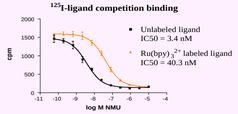
MESO SCALE BINDING ASSAY PRINCIPLE

The assay is based on detection of electrochemiluminescence (ECL). The peptide ligand was labeled with $Ru(bpy)_3^{2+}$. Cell membranes were immobilized to the surface of assay plates. Upon application of an electric current, the receptor-bound $Ru(bpy)_3^{2+}$-ligand undergoes an oxidation-reduction cycle in the presence of a co-reactant tripropylamine (TPA) and emits light. Signal is only generated when the $Ru(bpy)_3^{2+}$ label is in close proximity to the electrode, thus discriminating the bound label from unbound and enabling a no wash, homogenous format. Inhibitors of binding are detected by a decrease in light intensity.

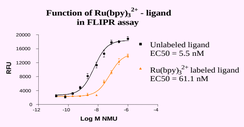


EFFECT OF $Ru(bpy)_3^{2+}$ -LABELING ON THE BIOLOGICAL ACTIVITY OF THE LIGAND

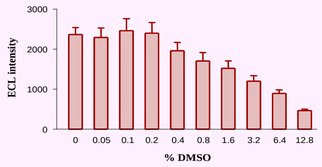
- **The labeling with $Ru(bpy)_3^{2+}$ caused >10 fold decrease of affinity in ^{125}I-ligand competition binding using SPA**



- **The labeling with $Ru(bpy)_3^{2+}$ caused >10 fold decrease of affinity in functional (FLIPR) assay**

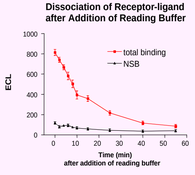


EFFECTS OF DMSO



The assay could not tolerate DMSO concentrations > 0.2%.

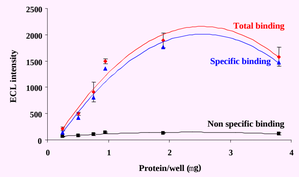
EFFECTS OF READING BUFFER



Time (min)	% remaining
0	100.0
2	95.1
4	82.6
6	70.2
8	61.4
10	47.0
15	43.1
25	24.6
40	11.5
55	6.5

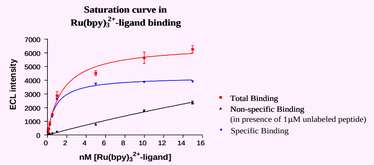
Upon addition of the TPA reading buffer, the labeled ligand rapidly disassociated from the receptor. Therefore, all readings were made between 1 and 2 minutes after the addition of reading buffer.

TITRATION OF MEMBRANE PROTEINS



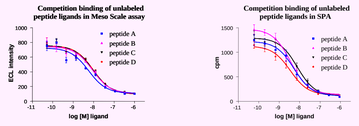
Specific binding was linear for up to 1 mg of membrane protein.

SATURATION BINDING



Kd (nM) = 0.89 ± 0.14, Bmax (pmol/mg protein) = 4.0

COMPETITION BINDING



Ki values (nM) of unlabeled peptide ligands

	Peptide A	Peptide B	Peptide C	Peptide D
Meso Scale	3.01	4.95	5.26	3.67
SPA	5.00	3.43	7.21	3.27

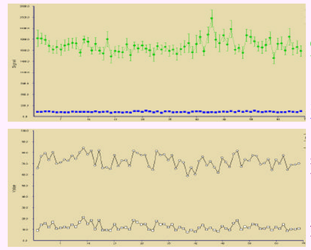
COMPARISON OF DIFFERENT ASSAYS

	Non Radioactive			Radioactive	
	DELFIA	FP	MSD	SPA - LeadSeeker	SPA - TopCount
Labeling molecule	Europium	Bodipy-TMR	$Ru(bpy)_3^{2+}$	^{125}I	^{125}I
Effects of labeling*	lost activity	less active	less active	no effect	no effect
Assay Window	N/A	1.4	> 10	5	> 10
Z'	N/A	< 0	0.7	0.5	0.7
Protein (µg/well)	N/A	10	1	5	10
Throughput (reading time: min/plate)	N/A	2	2	5	40

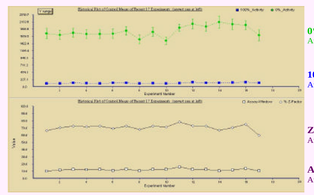
* Effects of labeling were measured using FLIPR assay for function (EC50) and 125I competition binding (IC50).

SCREEN PERFORMANCE

Typical daily results summary (70 plates)



Overall screen performance (summary of 1000 plates)



CONFIMED HITS WITH THEIR ACTIVITIES IN BOTH METHODS

Compound	Meso Scale % Activity	% Light Quench	SPA % Activity
A	51.21	< 1	< 40
B	48.35	10	53.68
C	52.12	18.1	< 40
D	73.51	27.4	< 40
E	56.37	8.6	< 40
F	68.65	25.7	66.34
G	44.38	13.7	< 40
H	42.13	20.3	< 40
I	48.22	9.5	< 40
J	55.88	13.5	< 40

- Data shown above were averages of triplicates.
- All hits showed minimal interference with ECL intensity.
- Meso Scale method appeared to be more sensitive in identifying actives.

RESULTS

- **Using Meso Scale binding assay, screened a total of 308,613 compounds.**
- **Good assay performance with assay window > 10 and Z' > 0.7.**
- **Throughput was 100 plates (384-well) per day, one person, one Sector HTS reader.**
- **267 primary hits identified using the cutoff of 40% inhibition and statistically different from plate median. Hit rate = 0.087%.**
- **10 actives confirmed in retest. Confirmation rate = 3.7%.**

CONCLUSION

- **A robust receptor-ligand binding assay using Meso Scale technology has been established and used in high throughput screening for inhibitors of a G protein-coupled neuropeptide receptor.**
- **Among the methods evaluated, the non-radioactive Meso Scale technology is the most sensitive and highest throughput method for receptor-ligand binding.**

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