

SelectScreen™ Cell-based GPCR Profiling Service: Compound Screening with the κ Opioid Receptor Utilizing Different Cell-Based Assay Platforms.

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Abstract

GPCRs are a common target for drug discovery. Assays for high-throughput screening have typically revolved around receptor binding, guanine nucleotide binding, and second messenger assays measuring intracellular cAMP and calcium levels. Protein redistribution is a new screening platform for GPCRs and involves the recruitment of β-arrestin to phosphorylated receptors. The introduction of ligand-induced differential signaling in which ligands have intrinsic efficacy for activating a receptor's downstream signaling cascades through multiple pathways, such as β-arrestin recruitment and second messenger signaling, has increased the complexity of GPCR pharmacology. The ability to characterize compounds in multiple screening platforms is needed for the identification of ligand-induced differential signaling. The SelectScreen™ GPCR profiling service utilizes the Tango™ and GeneBLAzer® cell lines containing the β-lactamase reporter gene technology which are dependent on β-arrestin recruitment and second messenger signaling respectively. These assays offer the ability to easily test differential signaling of compounds utilizing a single assay readout. Screening compound signaling through various mechanisms downstream of receptor-ligand binding should allow for better characterization of compound potency and efficacy. This poster will profile a set of compounds with the κ opioid receptor utilizing different cell-based assay platforms.

Figure 1 – GeneBLAzer® and Tango™ Assay Technologies

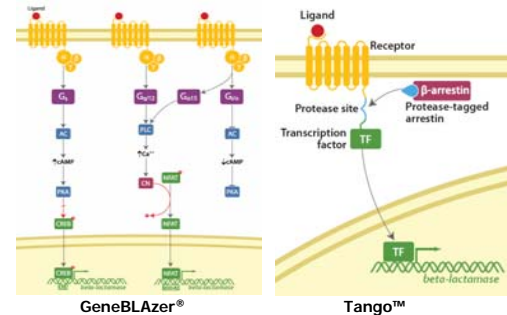
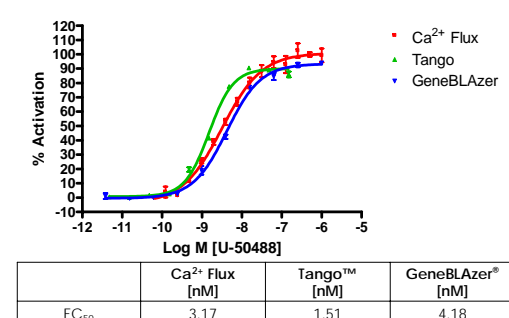


Figure 1: The GeneBLAzer® GPCR cell-based assays capture the downstream signaling through second messenger signaling pathways. Upon ligand binding and conformational change of the GPCR, one of 3 heterotrimeric G-proteins is activated and a G-protein dependent signaling cascade begins. Signaling through the G_{αi} G-protein leads to an increase in cAMP production and downstream activation of the cAMP response element (CRE). Signaling through the G_{αq} G-protein initiates the calcium flux signaling cascade and is captured by either the phosphorylation of the Nuclear Factor of Activated T-cells (NFAT) response element or by monitoring intracellular calcium flux. Signaling through the G_{αs} G-protein leads to a decrease in cAMP concentrations in the cell and is difficult to capture using cell based assays. Therefore the G_{αs} coupled signaling is redirected through the G_{αq} coupled pathway by using either promiscuous (G_{αs}) or chimeric G-proteins (G_{αs}/G_{αq}). GeneBLAzer® assays detect receptor activation through the beta-lactamase expression is measured by the addition of the LiveBLAzer™-FRET B/G substrate.

The Tango™ GPCR cell-based assays utilize a proprietary platform based upon receptor desensitization triggered by agonist binding to specific GPCRs. The GPCR of interest is linked to a non-native transcription factor by a TEV protease site. Agonist binding to the GPCR triggers the recruitment of protease-tagged arrestin proteins to the activated receptor. The recruited protease-tagged arrestin cleaves the TEV protease site. This cleavage releases the non-native transcription factor, which immediately enters the nucleus bypassing additional signaling intermediates. Once in the nucleus the transcription factor directly regulates transcription of a beta-lactamase reporter construct, which is measured upon addition of the LiveBLAzer™-FRET B/G substrate.

Figure 2 – Platform Comparison: Activation of OPRK1 by U-50488



GeneBLAzer® and Tango™ Assays: OPRK1-Gqo5-NFAT-bla CHO-K1 (GeneBLAzer®) and OPRK1-bla U2OS (Tango™) cells were plated at a density of 10,000 cells/well in assay media and incubated overnight at 37°C. The following day the plates were treated with a dose response of U-50488 and incubated for 5 hours at 37°C. The plates were then loaded at room temperature for 2 hours with the LiveBLAzer™-FRET B/G substrate. The plates were read with a Tecan Safire® and the calculated blue:green ratios (n=2) were graphed as % Activation, normalized to the full agonist control (100%) and the cell only control (0%).

Ca²⁺ Flux (Fluo4-NW) Assay: OPRK1-Gqo5-NFAT-bla CHO-K1 cells were plated at a density of 10,000 cells/well in assay media and incubated overnight at 37°C. The assay media was aspirated from the cells, and 25 μL of Fluo4-NW fluorescent dye was added to the cells and incubated at 37°C for 30 minutes, followed by an additional 30 minutes at room temperature. The assay was performed using the Hamamatsu FDS5. 5 μL of the U-50488 dose response was added to the cells with fluorescent readings taken every second for 3 minutes. The maximum minus minimum RFUs obtained for the U-50488 dose response (n=6) were graphed as % Activation normalizing the maximum-minimum values to the full agonist control (100%) and cell only control (0%).

The EC₅₀ values obtained for U-50488 in each of the three different cell-based assay platforms were comparable with EC₅₀ values of 3.17 nM (Ca²⁺ flux), 1.51 nM (Tango™), and 4.18 nM (GeneBLAzer®).

Table 1 – κ Opioid Receptor Agonist Panel

Compound	Ca ²⁺ Flux [nM]	Tango™ [nM]	GeneBLAzer® [nM]
Salvinorin A	0.717	0.92	6.48
U-69593	8.40	2.86	9.30
ICI-199.441	0.099	0.048	0.11
BRL52537	0.495	0.56	1.94

Table 1: A small panel of known κ opioid receptor agonists was obtained to compare the potency of these compounds in each of these cell-based platforms. The beta-lactamase reporter gene and Calcium Flux assays were performed as described above (Figure 2). The relative EC₅₀ values obtained in each of the assay platforms are indicated above.

In each of the three technologies the same rank order potency for the above compounds was maintained. The EC₅₀ values obtained with the assays performed using OPRK1-Gqo5-NFAT-bla CHO-K1 cells (Ca²⁺ flux and GeneBLAzer®) were comparable with the exception of Salvinorin A which had a lower EC₅₀ value in the Ca²⁺ flux assay. The Tango™ assay performed with the OPRK1-bla U2OS cells generated EC₅₀ values which were comparable to the values obtained in the Ca²⁺ flux assay with a slightly lower EC₅₀ value for U-69593 in the Tango™ assay.

Figure 3 – LOPAC¹²⁸⁰ Library Screen

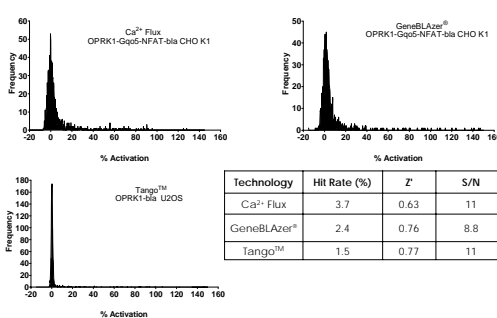


Figure 3: The LOPAC¹²⁸⁰ Library was screened in agonist mode against the κ opioid receptor in each of the aforementioned technologies: Ca²⁺ flux, GeneBLAzer®, and Tango™. Assays were performed as described previously and the LOPAC¹²⁸⁰ compounds were screened at a final concentration of 10 μM. The parental Gqo5-NFAT-bla CHO-K1 cell line was also screened in both Ca²⁺ flux and GeneBLAzer® technologies to account for non-specific activation. For each assay plate the data was normalized to the full agonist control (100%) and the cell only control (0%). Compounds which resulted in >50% activation were identified as "hits".

Frequency histograms are graphed above for each of the assays screened with the LOPAC¹²⁸⁰ library. The distribution of the compound activity are similar between the Ca²⁺ flux and GeneBLAzer® assays utilizing the OPRK1-Gqo5-NFAT-bla CHO-K1 cell line while the distribution of the OPRK1-bla U2OS cells utilizing the Tango technology is more tightly aligned around 0% activation. Despite similar signal to noise ratios the average Z' (n=4) increased with use of the beta-lactamase reporter gene.

Figure 4 – Agonist "Hit" Summary

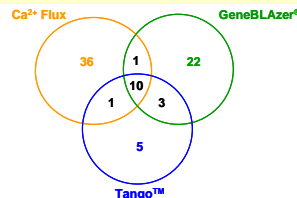


Figure 4: The "hits" (>50% activation) from the LOPAC¹²⁸⁰ library screen utilizing the Ca²⁺ flux, GeneBLAzer®, and Tango™ technologies where compared. The number of hits for each assay were 48, 36, and 19, respectively. 36 compounds were specific for the Ca²⁺ flux assay, 22 compounds for the GeneBLAzer® assay, and 5 compounds were specific for the Tango™ technology. 10 compounds from the LOPAC¹²⁸⁰ hit in all three of the technologies tested.

Results and Conclusions

- Rank order potency was maintained for the κ opioid agonists tested in all three technologies: Tango™ β-arrestin recruitment and GeneBLAzer® 2nd messenger signaling with both Ca²⁺ flux and reporter gene technologies.
- The Tango™ and GeneBLAzer® reporter gene technologies identified all κ opioid agonists within the LOPAC¹²⁸⁰ library while the Ca²⁺ flux assay missed one reported κ opioid receptor agonist.
- The Tango™ technology utilizing the OPRK1-bla U2OS cell lines had the fewest false positive "hits" of all three technologies evaluated.
- Use of the OPRK1-Gqo5-NFAT-bla CHO-K1 cells in the reporter gene beta-lactamase assay resulted in fewer non-specific "hits" compared to the Ca²⁺ flux assay performed with the same cell line.

Table 2 – "Hits" common to all three technologies

Compound	Class	Action	Subtype	Notes
S-(-) Eticlopride hydrochloride	Dopamine	Antagonist	D2	Potent and selective D2 dopamine receptor antagonist
GR-89696 fumarate	Opioid	Agonist	kappa	kappa opioid receptor agonist
(±) trans-U-50488 methansulfonate	Opioid	Agonist	kappa	Selective kappa opioid receptor agonist
ICI 204.448 hydrochloride	Opioid	Agonist	kappa	kappa opioid receptor agonist that does not cross the blood brain barrier
Loperamide hydrochloride	Opioid	Ligand		Meperidine congener which binds to opioid receptors. Ca ²⁺ channel antagonist
U-69593	Opioid	Agonist	kappa	Selective kappa opioid receptor agonist
U-62066	Opioid	Agonist	kappa	Highly selective kappa opioid receptor agonist. antitussive
(±) trans (1R,2R)-U-50488 hydrochloride	Opioid	Agonist	kappa	kappa opioid receptor agonist; less potent enantiomer of (±)-trans-U-50488
(-)-trans (1S,2S)-U-50488 hydrochloride	Opioid	Agonist	kappa	Potent kappa opioid receptor agonist; more potent enantiomer of (±)-trans-U-50488
U-101958 maleate	Dopamine	Antagonist	D4	Selective D4 dopamine receptor antagonist

Table 2: The 10 compounds with >50% activation in each of the three technologies are indicated above. The table contains the compound names, the receptor family with which they have been reported to act upon, whether the compounds are reported to function as an agonist or antagonist, if they are receptor subtype specific, as well as additional notes about the compounds identified. The LOPAC¹²⁸⁰ library has 13 compounds described as opioid agonists, 8 of which are classified as specific κ opioid receptor agonists. 7 of these compounds were identified as "hits" when the LOPAC¹²⁸⁰ library was screened in the three different technologies. Loperamide hydrochloride is identified as an opioid ligand and is reported to have high affinity for the μ opioid receptor with some activity toward the δ and κ receptors.

The one remaining κ opioid agonist, naloxone benzoylhydrazone, was 1 of the 3 "hits" common between the GeneBLAzer® and Tango™ technologies. Of the 5 compounds specific for the Tango™ technology, 2 derivatives of the sigma1 agonist (+)SKF-10,047, were classified as "hits" in the β-arrestin recruitment assay.

Table 3 – Follow-up dose response of non-opioid "hits"

Compound	Ca ²⁺ Flux [nM]	Tango™ [nM]	GeneBLAzer® [nM]
U-50488	10.7	2.15	6.37
U-101958 maleate	>10,000	>10,000	>10,000
Eticlopride Hydrochloride	>10,000	>10,000	>10,000

Table 3: The two compounds which gave >50% activation in each of the assays but are reported to be dopamine antagonists were tested in a follow-up dose response assay in each of the three technologies. The U-101958 and Eticlopride hydrochloride compounds were purchased and reconstituted in DMSO at 10 mM. A 1/2 log serial dilution was performed such that the top concentration of the dose response was 10 μM (the concentration used in the initial LOPAC¹²⁸⁰ screen). In each of the three technologies these two compounds did not show any activity. One possibility for this result could be due to a potential contamination in the original LOPAC¹²⁸⁰ plates used in the initial screens.